

TRANSFORMATION VON WIRBELTIEREN MITTELS
MIKROINJEKTION KLONIERTER GENE IN
BEFRUCHTETE EIZELLEN

Inaugural-Dissertation
zur Erlangung
der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von
SANDRO RUSCONI
von Locarno (TI)

begutachtet von Herrn Prof. Dr. W. Schaffner

Zürich ~~1982~~

1984

TRANSFORMATION VON WIRBELTIEREN MITTELS
MIKROINJEKTION KLONIERTER GENE IN
BEFRUCHTETE EIZELLEN

Inaugural-Dissertation
zur Erlangung
der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von
SANDRO RUSCONI
von Locarno (TI)

begutachtet von Herrn Prof. Dr. W. Schaffner

Zürich 1982

TRANSFORMATION VON WIRBELTIEREN MITTELS
MIKROINJEKTION KLONIERTER GENE IN
BEFRUCHTETE EIZELLEN

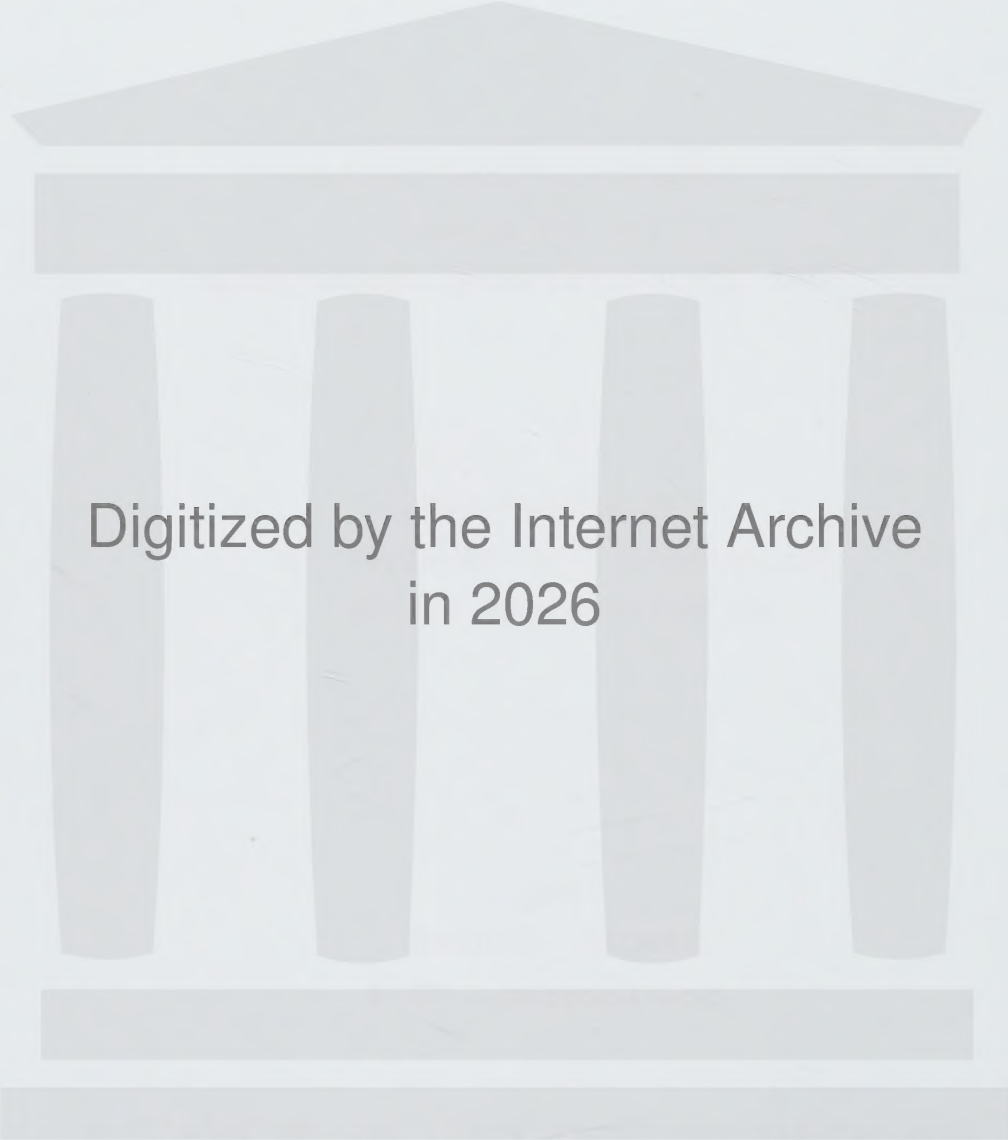
Inaugural-Dissertation
zur Erlangung
der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von
SANDRO RUSCONI
von Locarno (TI)

begutachtet von Herrn Prof. Dr. W. Schaffner

Zürich 1982



Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41507208_0004

Introduction

Chapter I

1. The Symbolic World

2. The Symbolic World as a System of Signs

3. The Symbolic World as a System of Values

Chapter II

4. The Symbolic World as a System of Signs

5. The Symbolic World as a System of Values

6. The Symbolic World as a System of Signs

Chapter III

7. The Symbolic World

8. The Symbolic World

9. The Symbolic World

10. The Symbolic World

11. The Symbolic World

12. The Symbolic World

13. The Symbolic World

14. The Symbolic World

15. The Symbolic World

16. The Symbolic World

17. The Symbolic World

18. The Symbolic World

19. The Symbolic World

20. The Symbolic World

21. The Symbolic World

22. The Symbolic World

23. The Symbolic World

24. The Symbolic World

25. The Symbolic World

26. The Symbolic World

27. The Symbolic World

28. The Symbolic World

29. The Symbolic World

30. The Symbolic World

Nature speaks in symbols and in signs

(John Greenleaf-Whittier, 1860)

ZUSAMMENFASSUNG	ii
EINLEITUNG	1
1) "Surrogate Genetics"	1
2) Warum will man ganze Organismen transformieren ?	2
3) Wie kann man Organismen transformieren ?	4
DISKUSSION	7
1) Ergebnisse mit dem Krallenfrosch <u>Xenopus laevis</u>	7
2) Transformation lebender Mäuse	9
SCHLUSSFOLGERUNGEN UND PERSPEKTIVEN	11
LITERATUR	13

ARBEIT Nr. 1, aus: Proceedings of the National Academy
of Sciences (USA) Vol. 78, pp. 5051-5055,

"Transformation of Frog Embryos with a Rabbit
 β -Globin Gene"

S. Rusconi and W. Schaffner (1981) 17

ARBEIT Nr. 2, to be submitted to Nature

"Cloned Genes Injected into Xenopus Zygotes Remain
Unmethylated and Result in Genetically Mosaic Organisms"

S. Rusconi and W. Schaffner (1982) 23

ARBEIT Nr. 3, to be submitted to Nucleic Acids Research

"Transformation of Mice with an in vitro Mutated
Mouse α -Globin Gene"

S. Rusconi and W. Schaffner (1982) 43

Z U S A M M E N F A S S U N G

Diese Arbeit beschreibt die erfolgreiche Transformation eukaryontischer Organismen (von Fröschen und Mäusen) durch Mikroinjektion klonierter Gene in befruchtete Eizellen.

In einer ersten Serie von Experimenten (Publikationen 1 und 2) wurde das Schicksal und die Expression eines in Zygoten des Krallenfrosches Xenopus laevis injizierten β -Globingens des Kaninchens untersucht. Das fremde Gen wurde während der Entwicklung repliziert und führte zu transformierten Fröschen, die Mosaiktiere aus transformierten und nicht-transformierten Organen darstellten. Die Expression des heterologen β -Globingens blieb auf die Periode der Furchungsteilungen beschränkt, in der die Mehrheit der Kaninchengene extrachromosomal vorlag. Unter Beibehaltung seines unmethylierten Status lag das fremde Gen aller Wahrscheinlichkeit nach in späteren Entwicklungsstadien in integriertem Zustand vor. Dabei konnte keine Expression mehr beobachtet werden. Die Konsequenzen dieses interessanten Befundes werden in der Publikation 2 ausführlich diskutiert.

Im zweiten Teil der Arbeit (Publikation 3) wird die Transformation von Mäusen mittels eines in vitro mutierten homologen Maus- α -Globingens beschrieben. Das Gen wurde in der nicht-translatierten 3'-Region durch Einfügung von DNA-Oligonukleotiden so modifiziert, dass eine Unterscheidung von endogenen α -Globingenen möglich war. Experimentell konnte nachgewiesen werden, dass durch die vorgenommenen Modifikationen (a) die korrekte Expression des Gens in Zellkulturen nicht beeinflusst wird, und (b) eine einfache Unterscheidung auf der DNA- (in transformierten Mäusen) und RNA-Ebene (in Rekonstruktionsexperimenten) von exogenen und endogenen α -Globinsequenzen selbst bei ungünstigem Verhältnis (z.B. bei einem 20-fachen Ueberschuss von Wildtyp-mRNA) gewährleistet ist.

Diese zwei Systeme der Organismentransformation (Frosch und Maus) und die Vor- bzw. Nachteile dieser Techniken werden in der Einleitung ausführlich diskutiert.

Herrn Prof. W. Schaffner, unter dessen Leitung diese Arbeit entstand, danke ich herzlich für alle Anregungen und hilfreichen Diskussionen. Ebenfalls möchte ich Herrn Prof. M.L. Birnstiel für die mir gewährte grosszügige Unterstützung herzlich danken.

Ohne meine vielen Kollegen, die mir bei der Uebersetzung halfen, und viele Impulse durch Diskussionen und Ratschläge gegeben haben, wäre die vorliegende Arbeit in diesem Umfang nicht zustande gekommen. Auch ihnen möchte ich an dieser Stelle von Herzen danken.

* * *

E I N L E I T U N G

1) "SURROGATE GENETICS"

Bei (nahezu) allen Organismen ist die Desoxyribonukleinsäure (DNA) der Träger der Erbinformation. Eine Anzahl struktureller und biochemischer Eigenschaften¹ machen die DNA zum geeigneten Molekül für die Speicherung und Reduplikation der Erbinformation. In Eukaryoten ist eine Gruppe von RNA-Polymerasen² für die Überschreibung (Transkription) der Erbinformation der Gene von der DNA in Ribonukleinsäure (RNA) verantwortlich. RNA-Polymerase I transkribiert die Gene für ribosomale RNA, RNA-Polymerase II die Protein-kodierenden Gene, also die überwiegende Mehrzahl aller Gene, und RNA-Polymerase III transkribiert im wesentlichen die (kleinen) tRNA- und 5S RNA-Gene. Die Transkripte der Protein-kodierenden Gene sind mehreren Processing-Schritten unterworfen (wie dem 'capping', 'splicing' und der Polymerisation der 3'-Poly A-Sequenz), werden aus dem Kern ins Cytoplasma transportiert und schliesslich in Proteine übersetzt. Die Regulation der Genexpression kann auf jedem dieser Schritte erfolgen³. Meist wird schon die Initiation der Transkription kontrolliert.

Die Fortschritte der rekombinanten DNA Technik haben die Strukturanalyse vieler eukaryontischen Gene ermöglicht. Wie schematisch in Abb. 1 dargestellt, kann die DNA aus einem höheren Organismus mit Restriktionsendonukleasen fragmentiert und in bakterielle Vektoren inkorporiert werden (s. Abb. 1b). Jedes dieser DNA-Fragmente kann für sich allein in einer Bakterienkultur amplifiziert (d.h. "kloniert") werden (s. DNA-Klonierung, Abb. 1b). Bakterienklone, die die gewünschte rekombinante DNA enthalten, werden meist durch Hybridisierung mit einem radioaktiv markierten Genfragment, der sog. "Sonde", identifiziert (s. Abb. 1c). Aus solchen DNA-Klonen

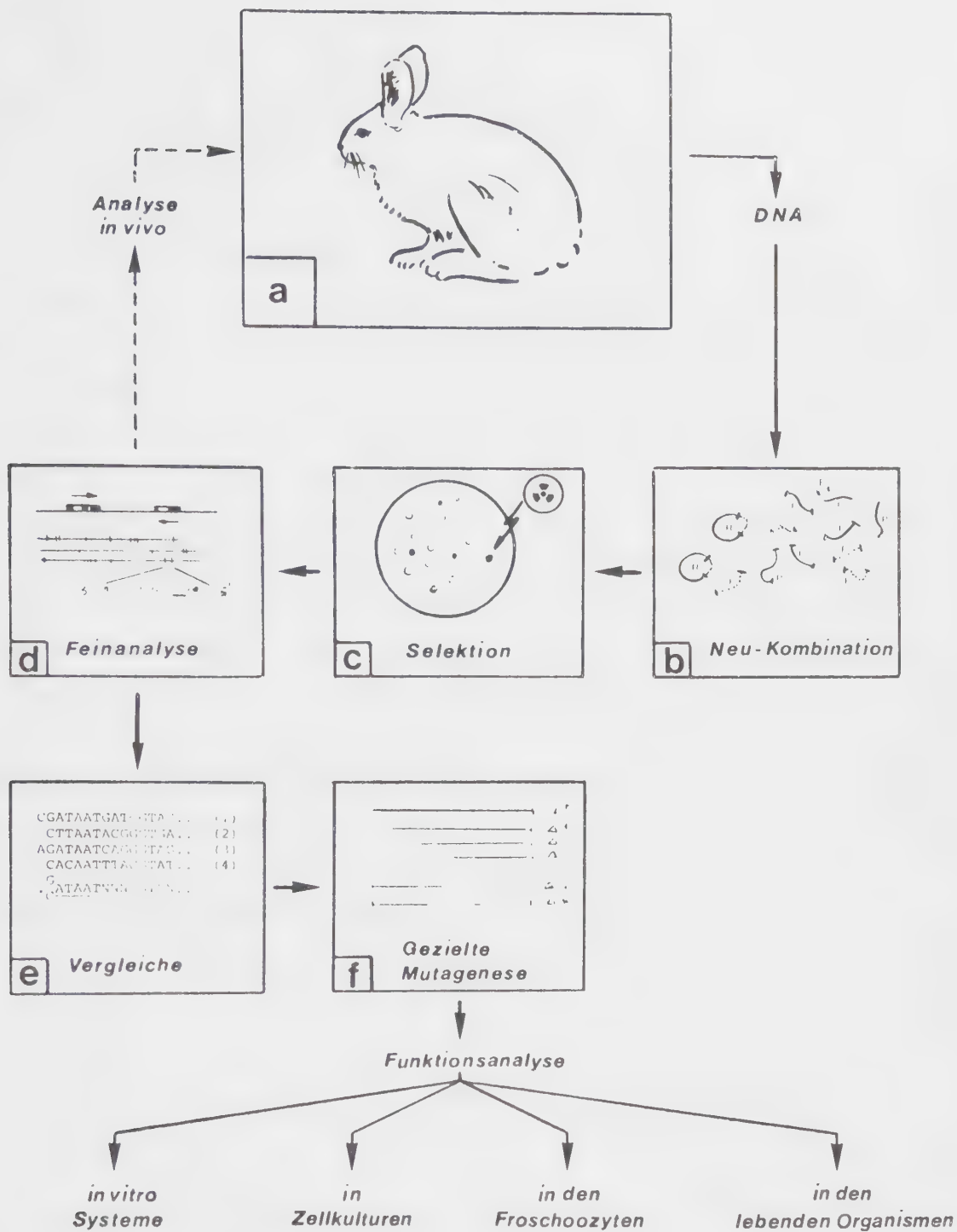


Abbildung 1

" Surrogate Genetics "

(Erklärungen im Text)

können grosse Mengen des betreffenden Gens isoliert und für weitere Analysen verwendet werden (s. Abb. 1d). Sehr viele Gene sind dank dieser Klonierungstechnik isoliert und im Detail charakterisiert worden. Ein Vergleich verschiedener Gene (Fig. 1e) hat gezeigt, dass gewisse Kontrollabschnitte im Verlaufe der Evolution kaum verändert wurden, so zum Beispiel der Sequenzabschnitt der Transkriptionsinitiationsstelle, die Intron-Exon-Übergangsstellen und die Region vor der Poly A-Sequenz. Damit ergab sich auch die Möglichkeit, solche sog. "konservierten" Abschnitte durch Insertion, Deletion oder Substitution zu mutieren und die funktionellen Auswirkungen zu untersuchen (s. Abb. 1f). Derartige Manipulationen sind als "Surrogate genetics"⁴ oder als "Ersatz-Genetik" bezeichnet worden. Eine Anzahl von in vitro- und in vivo-Funktionstests⁵ wurden entwickelt, um die Aktivität von Wildtyp- und mutierten Genen zu vergleichen (s. Abb. 1, unten). Obwohl derartige Experimente erst seit wenigen Jahren durchgeführt wurden, konnten damit bereits wichtige Erkenntnisse erhalten werden. So wurden z.B. mehrere DNA-Sequenzabschnitte identifiziert, die bestimmten Transkriptionssignalen entsprechen.

2) WARUM WILL MAN GANZE ORGANISMEN TRANSFORMIEREN ?

Die Kontrolle der Genexpression während der Embryonalentwicklung ist weitgehend unverstanden. Die eleganten Methoden der "Surrogate genetics" haben uns viele Informationen über die DNA-Sequenz der Gene und über DNA-Abschnitte, die essentiell für die Transkription sind, geliefert. Die sog. "TATA"-'box',⁶ eine in der Evolution kaum veränderte (also "konservative")

Sequenz, befindet sich ungefähr 30 Nukleotide 5' vor der Transkriptionsinitiationsstelle. Dieses Sequenzmotiv ist äusserst wichtig für die Wahl der korrekten Initiationsstelle durch die RNA-Polymerase II. In vivo Experimente haben gezeigt,⁶ dass nach Deletion der TATA-'box' statt der korrekten Startstelle eine Anzahl neuer Initiationsstellen benutzt werden. Die "konservierten" DNA-Sequenzen an den Intron-Exon-Uebergängen und vor dem Polyadenylierungsort sind wichtig für die korrekte Synthese ("Reifung") der Transkripte. Diese drei Sequenzmotive, die TATA-'box', die Sequenzabschnitte an den Intron-Exon-Uebergängen sowie vor dem Polyadenylierungsort, kommen in den meisten eukaryontischen Genen vor.⁴ Es ist deshalb wenig wahrscheinlich, dass sie für die zell- und entwicklungsspezifische Regulation der Genaktivität verantwortlich sind. Wahrscheinlicher ist, dass DNA-Sequenzen 5' vor dem TATA-Sequenzmotiv für solche Regulationsvorgänge eine Rolle spielen.

Genspezifische Promotor-Komponenten, sog. "upstream or far upstream sequences", sind an mehreren Genen identifiziert worden, so z.B. am Histon-H2A-Gen des Seeigels⁷ *Psammechinus*, im β -Globin-Gen des Kaninchens⁸ oder im Thymidinkinase-Gen des Herpesvirus.⁹ Es ist jedoch z.Z. unbekannt, ob solche "upstream sequences" für die gewebe- und entwicklungsspezifische Regulation der Genaktivität genügen. Es gibt zumindest Hinweise, dass die Genaktivität von noch weiter entfernten¹⁰ DNA-Abschnitten in cis beeinflusst werden kann.

Im allgemeinen wird die DNA-Sequenz der Gene während der Zelldifferenzierung nicht verändert. Eine bekannte Ausnahme davon stellen die Immunglobulin-Gene¹¹ dar. Allerdings gibt es eine Reihe anderer biochemischer Modifikationen, die über Zellgenerationen hinweg weitergegeben können, jedoch zell- und entwicklungsspezifisch verändert werden. So z.B. bestimmte DNA-Methylierungsmuster,¹² spezifische DNaseI-hypersensitive Chromatinregionen,¹³ lokale Akkumulation von "high mobility group proteins" (HGM-Proteine),¹⁴ von Ubiquitin¹⁵ oder bestimmten Histon-

¹⁶varianten. Die Signale für die zell- und gewebespezifischen Veränderungen müssen in genau definierter Art und Weise in der DNA manifestiert sein. Da es z.Z. unmöglich ist, den Gesamt-
ablauf der Zelldeterminierungs- und Zelldifferenzierungsprozesse in vitro nachzuvollziehen, ist man darauf angewiesen, Gene während der Gesamtentwicklung eines intakten Organismus zu analysieren. Es ist deshalb z.Z. die Methode der Wahl, modifizierte Gene in befruchtete Eizellen einzuführen und ihr "Schicksal", vor allem ihre Aktivität, während der Entwicklung des Organismus zu untersuchen. Dadurch wird es möglich, die funktionelle Bedeutung von DNA-Signalsequenzen für die Regulation der Genexpression in ihrer Gesamtheit zu analysieren und zu charakterisieren.

3) WIE KANN MAN ORGANISMEN TRANSFORMIEREN ?

¹⁷1944 transformierte Avery Pneumokokkus-Bakterien mit DNA und bewies damit, dass die DNA das genetische Material darstellt. Seit diesen bahnbrechenden Arbeiten ist immer wieder versucht worden, ¹⁸auch höhere Organismen mit DNA zu transformieren. Bevor man mit klonierten Genen arbeiten konnte, waren die Resultate solcher Experimente kaum interpretierbar. Die heutigen Techniken erlauben jedoch sowohl die Verwendung von exakt definierten DNA-Abschnitten für die Transformation als auch die genaue Analyse des Schicksals und der Expression der eingeführten Gene.

Der Ausdruck "Transformation" soll hier im weitesten Sinne gebraucht werden. Also auch für die Fälle, in denen die eingeführte DNA nicht exprimiert und somit der Phänotyp des Organismus nicht verändert wird.

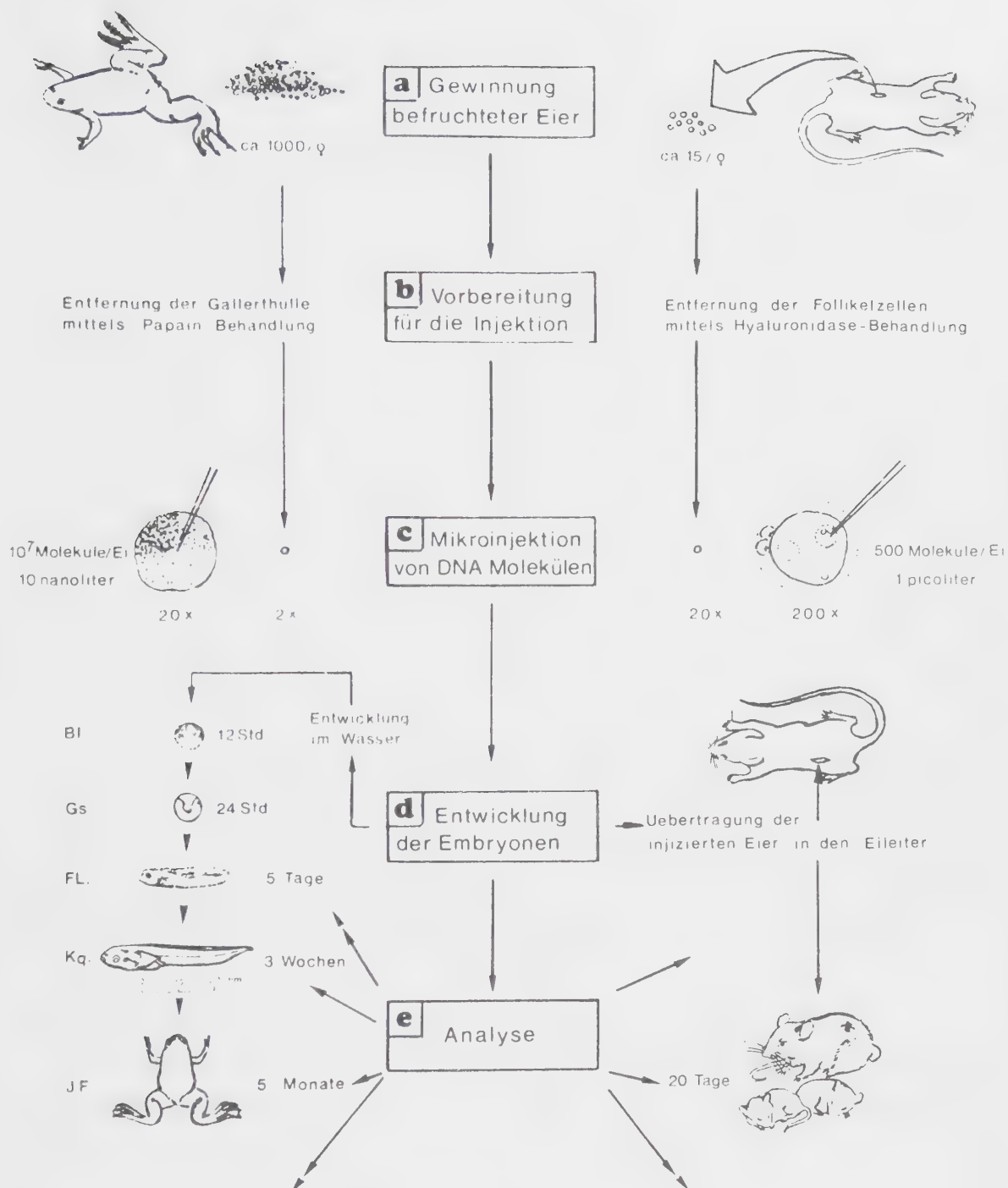


Abbildung 2 Methodik der Organismen-Transformation

Legende: BI., Blastula; Gs., Gastrula; FL., "feeding larva"; Kq., Kaulquappe; JF., junger Frosch;

Weitere Erklärungen im Text.

Die drei nachfolgenden, z.T. bereits publizierten Arbeiten haben Transformationsexperimente mit Fröschen (Arbeiten Nr.1 und 2) und Mäusen (Arbeit 3) zum Inhalt. Die dazu notwendigen experimentellen Techniken sind in Abb.2 dargestellt und sollen hier kurz erläutert werden.

1.) Mikroinjektion von befruchteten Xenopus-Eizellen:

Nach der Injektion menschlichen Gonadotropins legt ein Froschweibchen Tausende von Eiern (Abb.2a, linke Seite). Um eine synchrone Entwicklung der Eier zu gewährleisten, werden diese Eier in vitro befruchtet. Nach weiterer Vorbehandlung (s. Abb. 2b) sind sie bereit zur Injektion. Dazu lässt man die Eizellen auf ein Nylonnetz von 1 mm Maschenweite am Boden einer Petrischale absinken. Da der Kern im dotterhaltigen, stark pigmentierten Ei nicht sichtbar ist, wird die DNA (ca. 10^7 DNA-Moleküle in 10 μ l) ins zentrale Cytoplasma der Eizelle bei 20facher Vergrößerung unter einem Auflicht-Stereomikroskop mit einer Glas-Mikropipette von ca. 25 μ m Durchmesser injiziert. Die Eizellen entwickeln sich gut in Leitungswasser bei 18°C. Abb. 2d zeigt einige Entwicklungsstadien des Krallenfrosches *Xenopus laevis*.

2.) Mikroinjektion von befruchteten Maus-Eizellen:

Die Mikroinjektion von Mauseizellen erfolgt in etwas modifizierter Art und Weise. Mäuse-Weibchen werden mit Männchen zusammengebracht, und am darauffolgenden Morgen ist eine erfolgte Kopulation am Vaginalpfropf ersichtlich. Am frühen Nachmittag können die befruchteten Eizellen (10 bis 20 pro Weibchen) aus dem Eileiter isoliert werden (Abb. 2a, rechte Seite). Die Eizellen mit einem Durchmesser von ca. 80 μ m werden bei 400facher Vergrößerung unter einem Durchlicht-Mikroskop injiziert. Dabei wird die Eizelle mit einer feinen Pipette angesaugt und festgehalten. Eine zweite, extrem dünn ausgezogene Pipette von etwa 1 μ m Durchmesser, die Injektionspipette, enthält die DNA-Lösung und wird, wie ihr Name sagt,

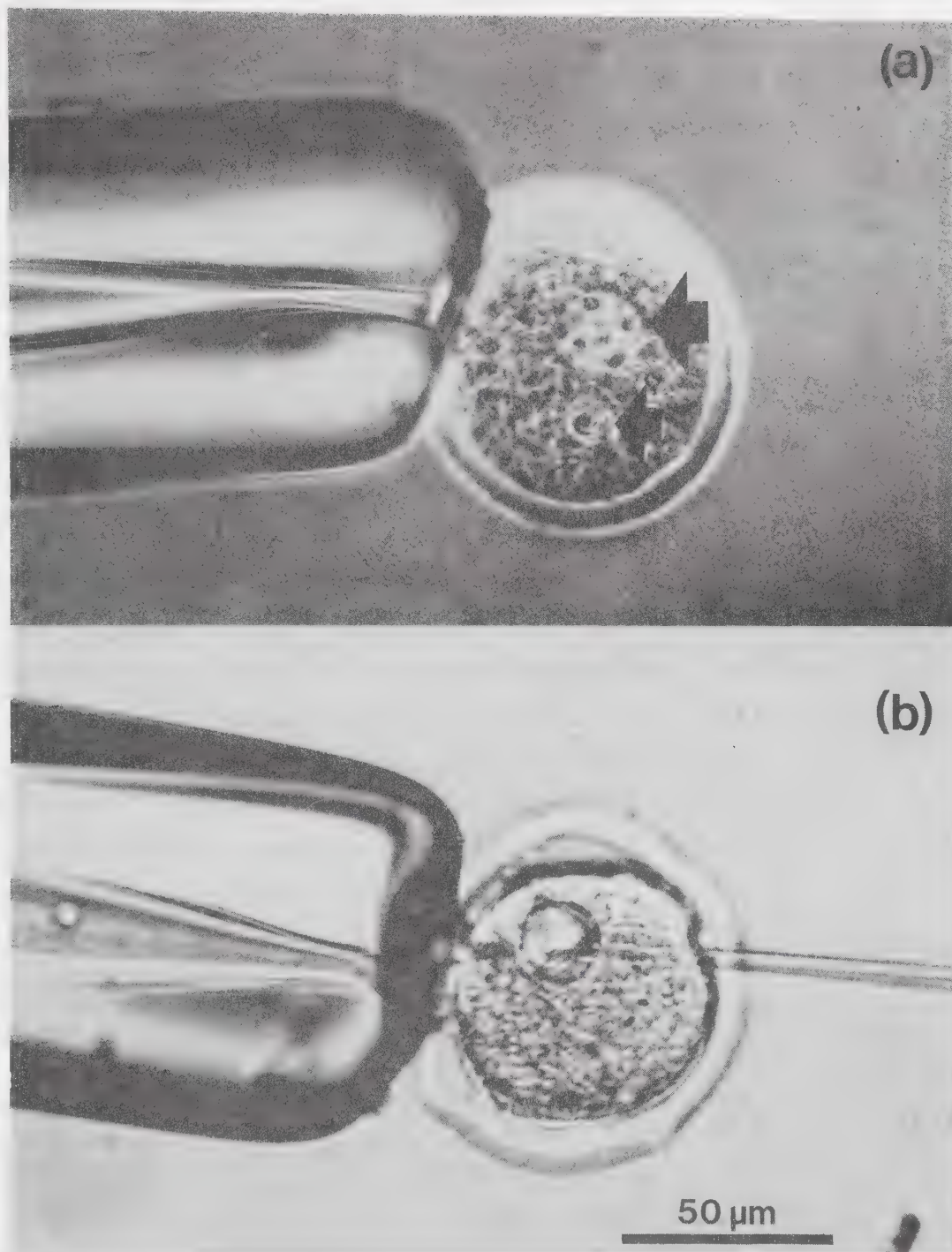


Abbildung 3 Mikroinjektion von DNA in befruchtete Maus-Eizellen

a) Eine Maus-Eizelle mit Pronuclei (Pfeile) vor der Injektion. (Vergrößerung, ca. 650 x).

b) Die Eizelle während der Injektion von ca. 1 picoliter DNA-Lösung (ca. 1000 DNA-Moleküle).

zur Injektion benutzt. Etwa 1 Picoliter DNA-Lösung , das heisst 500-2000 DNA-Moleküle, wird in den männlichen Pronucleus der Eizelle injiziert. Eine befruchtete Eizelle mit Pronuclei vor und während der DNA-Injektion (siehe Pfeile) ist in Abb. 3 abgebildet.

Die injizierten Eizellen werden einer scheinträchtigen Empfängermaus eingeführt (solche Empfängermäuse erhält man durch Verpaarung mit einem vasktomierten Männchen). Beide Schritte, die Injektion und die Rückführung in den Eileiter, verlangen experimentelles Geschick und Uebung. Der Aufwand lohnt sich jedoch u.a. deshalb, weil Mäuse im Gegensatz zu Fröschen eine sehr kurze Generationszeit von 50-60 Tagen (gegenüber 12 Monaten bei Fröschen) besitzen. Durch die Weitergabe der neuen Gene an nachfolgende Generationen entstehen aus den oft in der ersten Generation auftretenden "Mosaiktieren" genetisch homogene Organismen, die für viele weiterführende Versuche unentbehrlich sind.

D I S K U S S I O N

1) ERGEBNISSE MIT DEM KRALLenfROSch XENOPUS LAEVIS

(Arbeiten Nr. 1 und Nr. 2)

Für diese Serie von Experimenten habe ich ein kloniertes β -Globin-Gen des Kaninchens verwendet. Das Gen wurde als Teil von Plasmidchimären, die in einigen Fällen auch SV40-DNA enthielten, entweder direkt oder nach Entfernung der Plasmid-DNA in die Eizellen injiziert. Erstaunlicherweise wurde die injizierte DNA während der Replikation der Zell-DNA in den embryonalen Furchungsstadien zusammen mit dieser redupliziert. Die maximale Menge fremder extrachromosomaler β -Globin-DNA wurde im Gastrulastadium erreicht (siehe Abb.1, Arbeit Nr.2). Diese extrachromosomale DNA wurde später rasch abgebaut. Im Entwicklungsstadium 52 (3 Wochen alte Kaulquappe) waren die verbliebenen Kaninchen- β -Globin-Gensequenzen ausschliesslich in hochmolekularer Xenopus-DNA anzutreffen und wurden demnach aller Wahrscheinlichkeit nach stabil in ein (oder mehrere) Froschchromosom (en) eingebaut. Aus zusätzlichen Experimenten können wir schliessen, dass die Kaninchen- β -Globin-DNA in Form direkter Tandem-Repetitionseinheiten in das Genom der Kaulquappe inkorporiert und über die Metamorphose hinweg beibehalten wurde (s. Abb.3 in Arbeit Nr. 1)

Die Verteilung der DNA in jüngeren Xenopus-Fröschen wurden im Detail untersucht. Eine Analyse der Gewebe und Organe einzelner Individuen zeigte, dass die Frösche sog. genetische "Mosaike" mit ganz unterschiedlicher Verteilung der Kaninchen-Gene waren (siehe Abb. 2, Arbeit Nr.2). Ein Vergleich mehrerer Individuen ergab keinerlei Hinweise für den bevorzugten Einbau in ein bestimmtes Organ. Die Entstehung solcher Mosaiktiere lässt sich durch multiple unabhängige Integrationen der DNA erklären. Die in die Eizelle injizierte DNA ist offenbar in

die eine oder die andere Zelle eingebaut worden. Die Kaninchen- β -Globingene wurden nur so lange transkribiert, als sie in extrachromosomaler Form vorlagen (siehe Abb. A, Arbeit Nr.1). Interessanterweise startete die Transkription nur dann an der korrekten Initiationsstelle, wenn Plasmid-freie Kaninchen-DNA injiziert wurde. Dieser Befund stimmt mit anderen experimentellen Ergebnissen unseres Instituts überein (siehe auch Diskussion in Arbeit Nr.3), dass die Verknüpfung mit Plasmid-DNA zu Störungen in der Transkription eines eukaryontischen Gens führen kann.

Hingegen konnte (selbst nach Transformation mit Plasmid-freier DNA) keine Transkription des fremden Gens in Kaulquappen oder Fröschen, welche keine extrachromosomale Kaninchen-Gene mehr enthielten, festgestellt werden. Eine Analyse des Methylierungsmusters der Kaninchen-DNA (siehe Abb.3 der Arbeit Nr.2) zeigte, dass die stabil eingebauten β -Globingene kaum oder gar nicht methyliert waren. Dieser Befund ist insofern erstaunlich, da inaktive Gene bei Säugern und Vögeln meist stark methyliert sind (siehe dazu Kommentar zu Abb. 3 in Arbeit Nr.2). Es stellt sich damit die Frage, ob die negative Korrelation zwischen DNA-Methylierung und Genaktivität auch für Amphibien und nicht etwa nur für warmblütige Wirbeltiere gilt. Zumindest ist aus unseren Daten ersichtlich, dass ein Gen auch auf andere Art und Weise und nicht nur als Folge von DNA-Methylierungen inaktiviert werden kann. Weitere mögliche Gen-Inaktivierungsmechanismen werden in den Arbeiten 2 und 3 ausführlich diskutiert.

2) TRANSFORMATION LEBENDER MAEUSE

(Arbeit Nr. 3)

Die Transformation von Mäusen durch DNA-Injektion in Eizellen ist in den letzten zwei Jahren in mehreren Forschungsgruppen gelungen (siehe "Introduction" in Arbeit Nr.3). In fast allen Fällen stammten die verwendeten Gene von anderen Tiergattungen. Das erwies sich - wie in unseren Versuchen mit *Xenopus* - als vorteilhaft für die Identifikation der Gene und ihrer Transkripte im Wirtsorganismus. Die bisher erzielten Ergebnisse zur Expression dieser artfremden Gene sind jedoch wenig zufriedenstellend. Schon seit geraumer Zeit sind wir deshalb der Meinung, dass biologisch relevantere Experimente mit homologen Genen durchgeführt werden müssen.

Für unsere Experimente mit Mäusen benutzte ich ein von Prof. P. Leder und Mitarbeitern kloniertes α -Globin Gen der Maus. Um dieses Gen in transformierten Mäusen nachweisen zu können, wurden DNA "linker" in das nichttranslatierte 3'-mRNA-Segment des Gens eingefügt (siehe Abb. 1 in Arbeit 3). Diese zielgerichtete Mutation war - wie erwartet - äußerst nützlich zur Identifizierung der Transkripte dieses Gens (siehe Abb. 2). Die mutierten Gene wurden in *in vitro*-kultivierten HeLa Zellen übertragen und auf ihre funktionelle Aktivität getestet. Dabei zeigte sich, dass die mutierten Globin-Gene normal transkribiert und die Primärtranskripte einem normalen Processing unterzogen werden, aus dem Globin-messenger RNAs entstanden.

In meinen Versuchen erhielt ich vier transformierte Mäuse. Zur Identifizierung dieser Transformanten waren die 'linker'-DNA-Sequenzen mit ihren zusätzlichen Restriktionsorten nützlich, da dadurch die neuerworbenen Gene von den normalerweise vorhandenen unterschieden werden konnten (s. Abb. 3 in Arbeit 3).

Sollte sich herausstellen, dass die mutierten Gene auch in den Blutzellen der transformierten Mäuse

transkribiert und exprimiert werden, so kann unser Versuchsansatz wohl allgemein angewandt werden. Die Methode, spezifische und diagnostische Veränderungen in "neutrale" Positionen eines Gens einzuführen, ist weit weniger aufwendig als die Suche nach natürlich vorkommenden polymorphen Genvarianten. Das Verfahren konnte auch auf die Identifizierung von Proteinen ausgedehnt werden, indem man durch Mutation der DNA zur Diagnose geeignete, jedoch in ihrer Funktion neutrale Aminosäuresubstitutionen in ein bestimmtes Protein einführt.

In Zukunft wird es sicherlich auch möglich sein, durch die Transformation von Genen derselben Spezies homologe Integrationen zu erhalten, wie zum Beispiel den Einbau eines mutierten α -Globingens in den α -Globingen-Locus im Chromosom Nr.11 der Maus (siehe dazu Diskussion in Arbeit Nr.3). Ein solcher Einbau in die homologe Position ist äusserst erstrebenswert, da damit klonierte und "nach Wunsch" mutierte Gene wieder in ihre natürliche Umgebung zurückgeführt werden könnten.

Es wird sich zeigen, welcher Weg im Detail eingeschlagen werden muss, um unser ehrgeiziges Ziel zu erreichen, nämlich DNA-Signalsequenzen zu identifizieren, die die entwicklungsspezifische Regulation der Gene steuern.

S C H L U S S F O L G E R U N G E N U N D P E R S P E K T I V E N

Trotz des ausserordentlichen Fortschritts der Molekularbiologie während der letzten Jahre blieb die Fragestellung, wie die Aktivität von Genen im Verlaufe der Zelldifferenzierung reguliert wird, weitgehend ungeklärt. Die in der vorliegenden Dissertationsschrift dargestellten Experimente sind ein Beispiel der bisher bereits unternommenen vielfältigen Anstrengungen, die äusserst effektiven Methoden des 'genetical engineering' zum Studium der Vielzahl komplizierter Reaktionsmechanismen zu benutzen, die die Entwicklung höherer Organismen und letztendlich auch des Menschen determinieren.

Das Endziel des genannten Vorhabens ist die Schaffung experimenteller Möglichkeiten, eines Tages auch auf molekularer Ebene die Vielfalt der Wechselbeziehungen analysieren zu können, die zwischen dem eukaryontischen Genom und seiner zellulären Umgebung existieren müssen, damit aus einer Zygote ein adulter vielzelliger Organismus entstehen kann. Wird sich das als möglich erweisen, dann sprechen die potentiellen Anwendungsmöglichkeiten dieser Forschung für sich selbst. Solche Möglichkeiten, wie z.B. die gezielte genetische Verbesserung des Ertrags von Nutztieren und Nutzpflanzen oder gar die biochemische Therapie von Krebserkrankungen scheinen zur Zeit noch in weiter Ferne zu liegen. Doch mag man sich dabei vergegenwärtigen, dass vor nicht mehr als 10 bis 15 Jahren die

Isolierung einzelner Protein-kodierender Gene eukaryontischer Organismen als unmöglich erschien, während sie heute bereits zum Bestandteil des Methodenkatalogs jedes molekularbiologischen Laboratoriums und somit zu einer "Routinemethode" geworden ist.

L I T E R A T U R

- 1) - Zimmerman , S.B. (1982) Annu. Rev. Biochem.
Vol. 51, pp. 395-428
- Sheinin, R., Humbert, J. and Pearlman, R.E. (1978)
Annu. Rev. Biochem. Vol. 47, pp. 277-316
- Watson, J.D. (1977) Molecular Biology of the Gene,
3rd ed, W.A. Benjamin, Menlo Park, Cal.
- 2) - Chambon, P. (1975) Annu. Rev. Biochem. Vol. 44,
pp. 613-638
- 3) - Darnell, J.E. Jr (1982) Nature 297, 365-370
- Revel, R.M. and Groner, Y. (1978) Annu. Rev. Biochem.
Vol. 47, pp. 1079-1096)
- Perry, R.P. (1976) Annu. Rev. Biochem.. Vol. 45,
pp. 605-630
- 4) - Birnstiel, M.L. and Chipchase, M. (1977) Trends
in Biochem. Sci. 2, 149-152
- Shortle, D., Di Maio, D. and Nathans, D. (1981)
Annu. Rev. Genet. 15, 265-294
- Breatnach, R. and Chambon, P. (1981) Annu. Rev.
Biochem. Vol. 50, pp. 349-384
- 5) - Kressmann, A. and Birnstiel, M.L. (1980) in:
Transfer of Cell Constituents into Eucaryotic Cells,
NATO Advanced Study Series A, eds. Celis, J.E., Gräs-
sman, A. and Loyter, A. (Plenum, New York) Vol. 31,
pp. 383-407
- 6) - Grosschedl, R. and Birnstiel, M.L. (1980) Proc.
Natl. Acad. Sci. (USA) 77, 1423-1426

- 7) - Grosschedl, R. and Birnstiel, M.L. (1982) Proc. Natl. Acad. Sci. (USA) 79, 297-301
- 8) - Dierks, P. van Ooyen, A., Marti, N. and Weissmann, C. (1981) Proc. Natl. Acad. Sci. (USA) 78, 1411-1415
 - Dierks, P., Wieringa, B., Marti, D. Reiser, J., van Ooyen, A., Meyer, F., Weber, H. and Weissmann, C. in: Developmental Biology Using Purified Genes, ICN-UCLA Symposia on Molecular & Cellular Biology, ed. D.D. Brown (New York: Academic Press) Vol. 23, pp. 347-366 (1981)
- 9) - Mc. Knight, S., Gavis, E.R., Kinsburg, R. and Axel, R. (1981) Cell 25, 385-398
- 10) - Maniatis, T., Fritsch, E.F., Lauer, J. and Lawn, R.M. (1980) Annu. Rev. Genet. 14, 145-178
 - Klar, A.J.S., Strathern, J.N., Broach, J.R. and Hicks, J.B. (1981) Nature 289, 239-241
 - Nasmyth, K.A. Tatchell, K., Hall, B.D. Astell, C. and Smith, M. (1981) Nature 289, 244-248
 - Weintraub, H. and Groudine, M. (1976) Science 193, 848-858
- 11) - Bentley, D.L. and Rabbits, T.H. (1980) Cell 24, 613-623
 - Blomberg, B., Traunecker, A., Eisen, H. and Tonegawa, S. (1981) Proc. Natl. Acad. Sci. (USA) 78, 3765-3769
 - Joho, R., Weissmann, I.L., Cole, J. and Hood, L (1980) Proc. Natl. Acad. Sci. (USA) 77, 1106-1110
- 12) - Felsenfeld, G. and Mc Ghee (1982) Nature 286, 602-603
 - Razin, A. and Riggs, A. (1980) Science 210, 604-610
- 13) - Weintraub, H., Larsen, A. and Groudine, M. (1980) Cell 24, 333-344
 - Weintraub, H., Beng, H., Groudine, M. and Graf, T. (1982) Cell 28 931-940

- Wood, W.I. and Felsenfeld, G. (1982) Jour. Biol. Chem. 257, 7730-7736
- Wu, C. and Gilbert, W. (1981) Proc. Natl. Acad. Sci. (USA) 78, 1577-1580
- 14) - Weisbrod, S. and Weintraub, H. (1979) Proc. Natl. Acad. Sci. USA 76, 630-634
- 15) - Levinger, L and Varshavsky, A. (1982) Cell 28, 375-385
- 16) - Simpson, R.T. (1981) Proc. Natl. Acad. Sci. (USA) 78, 6803-6807
- Cohen, L.H., Newrock, K.M. and Zweidler, A. (1975) Science 190, 994-997
- 17) - Avery, O.T., McLeod, C.M. and McCarty, M. (1944) J. Exp. Med. 79, 137 and ff.
- 18) - Sirotnak, F.M. and Hutchinson, D.G. (1959) Biochim. Biophys. Acta 36, 246 and ff.
- Fahmy, O.G. and Fahmy, M.H. (1965) Nature New Biology 207, 507-510
- Ledoux, L. and Huart, R. (1969) Jour. Mol. Biol. 43, 243-262
- Fox, A.S. and Yoon, S.B. (1970) Proc. Natl. Acad. Sci. (USA) 67, 1608-1615
- Vielkind, J., Vielkind, U. Grotthuss, E. and Anders, F. (1971) Experientia 27, 347-348
- Germeraad, S. (1976) Nature 262, 229-231

ARBEIT Nr. 1

Transformation of frog embryos with a rabbit β -globin gene

(gene transfer/microneedle injection/DNA replication/gene regulation)

SANDRO RUSCONI AND WALTER SCHAFFNER

Institut für Molekularbiologie II der Universität Zürich, Höggerberg, CH-8093 Zürich, Switzerland

Communicated by V. Prelog, April 28, 1981

ABSTRACT In order to study the fate and possible expression of foreign DNA during embryogenesis of the frog *Xenopus laevis*, we have injected a rabbit β -globin gene into fertilized *Xenopus* eggs. Frog embryo DNA was extracted at various stages of development, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled β -globin recombinant plasmid DNA. It was found that the injected DNA replicated extrachromosomally, reaching, at gastrula stage, a level equivalent to a 10- to 200-fold amplification of input DNA. At later stages, a majority of the foreign DNA was degraded, but a small fraction was maintained. Six-week-old tadpoles as well as six-month-old frogs contained an average of 3-10 copies of the rabbit globin gene per cell. Most of these persisting globin genes were present as long tandem repeats and comigrated in agarose gel electrophoresis with high molecular weight *Xenopus* DNA. Analysis of globin gene expression by S1 nuclease mapping showed that the rabbit β -globin promoter was recognized in the frog embryo and that the transcripts were correctly spliced.

Oocytes and eggs of the frog *Xenopus laevis* have been used for many years as experimental systems for studying the translation of injected mRNAs (reviewed in ref. 1). More recently, microneedle injection of DNA into *Xenopus* oocytes has provided a functional test to analyze the biological activity of cloned genes (2-5), a concept named "surrogate genetics" (6). Unfertilized eggs and egg extracts are reported to promote replication of injected DNA (7-12). Fertilized eggs injected with DNAs (13, 14) or rabbit globin mRNAs (15) are able to continue developing into apparently normal tadpoles.

In the present studies, we have injected a genomic rabbit β -globin gene (16) into fertilized eggs of *Xenopus laevis* and have studied its fate throughout development. We have chosen the rabbit gene for the following reasons: (i) rabbit globin DNA can be identified by hybridization in the presence of a large excess of *Xenopus* DNA; (ii) intracellular rabbit β -globin protein is not toxic for *Xenopus* embryogenesis (15); and (iii) the molecular structure of the cDNA plasmid (ref. 17; recloned by F. Meyer, Zürich) and of the genomic gene (16, 18, 19) were known in detail. Our results indicate that the injected genes replicate and are correctly transcribed during early embryogenesis, and that they persist in young frogs.

MATERIALS AND METHODS

Injected DNAs. The genomic rabbit β -globin gene clone λ Ch4A-R β G1 was obtained from T. Maniatis, Pasadena, CA [refs. 16 and 18; referred to as the "genomic" clone, as distinguished from the "gnomic" Zürich clone independently isolated by C. Weissmann and his colleagues (20)]. The globin gene was excised from λ Ch4A-R β G1 and recloned as follows (cf. Fig. 1): (i) pP+, the 6.5-kilobase pair (kb) *Pst* I globin gene fragment,

was inserted into the *Pst* I site of the plasmid pBR322 (21, 22) in opposite orientation with respect to the β -lactamase gene; (ii) pSVK+, the 4.7-kb *Kpn* I globin gene fragment, was inserted into the single *Kpn* I site found in the simian virus 40 (SV40) "late" region of the previously constructed recombinant plasmid pBSV-early (23); (iii) pSVKd+, the same as pSVK+ but with two globin gene inserts in tandem; (iv) pSVKd-, as pSVKd+ but with two globin genes in the same orientation as the β -lactamase gene; (v) pJKd-, the 4.7-kb *Kpn* I globin gene fragment cloned as a dimer insert into the plasmid pJC-1 (pJC-1 is a derivative of pBR322 containing a *Kpn* I site instead of the *Eco*RI site, a gift of J. Jenkins, London); (vi) monomeric circles of the globin gene *Kpn* I fragment free of plasmid DNA were prepared as described for histone genes (24). Dimeric circles were made from the plasmid pJKd-*, a derivative of pJKd- in which the *Kpn* I site between the two globin genes had been destroyed by *Kpn* I cleavage, S1 nuclease digestion, and religation. Recombinant plasmids between pBR322 and globin gene DNA were constructed by standard cloning procedures (25, 26). All work involving recombinant plasmids was done under conditions conforming to the standards outlined in the National Institutes of Health guidelines for recombinant DNA research.

Microinjection. Artificially fertilized eggs of *X. laevis* were prepared as described by Billett and Wild (27). Fertilized eggs were decapsulated for 2 min in a solution of 3% (wt/vol) L-cysteine, 0.1% papain, and 0.23 M NaOH and then washed five times with 2 ml of fresh Barth's solution. Healthy fertilized eggs were injected within 1 hr, before the first cleavage event occurred. The microinjection technique was essentially the same as used for oocytes (2), except that the centrifugation step was omitted. Each egg was injected with a glass capillary needle from which there was a constant flow of DNA. The needle was inserted through the vitellin membrane at a 45° angle; unless otherwise stated, 250 pg of DNA (about 2×10^7 genes) in a volume of 10 nl was deposited within about 4 sec at the center of the egg. Two hours after injection, embryos were transferred from Barth's medium to sterile "aged" water (27) plus antibiotics (GIBCO) and incubated at 18°C.

Analysis of DNA and RNA. Samples of 5-10 embryos were collected immediately after injection and at the indicated stages of development according to Daudin (28). Embryos or tadpoles were homogenized at 0°C in 10 mM EDTA, 100 mM Tris-HCl (pH 7.5), 300 mM NaCl, proteinase K at 1 mg/ml, and 2% (wt/vol) sodium dodecyl sulfate. Frogs were treated the same way except that the gut was removed prior to homogenization. After a 30-min incubation at room temperature, the homogenate was extracted three times with phenol and two times with chloroform, and the nucleic acids were precipitated with 2 vol of ethanol. For Southern blotting (29, 30), DNA isolated from embryos at progressive stages of development was electrophoresed

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: kb, kilobase pairs; SV40, simian virus 40.

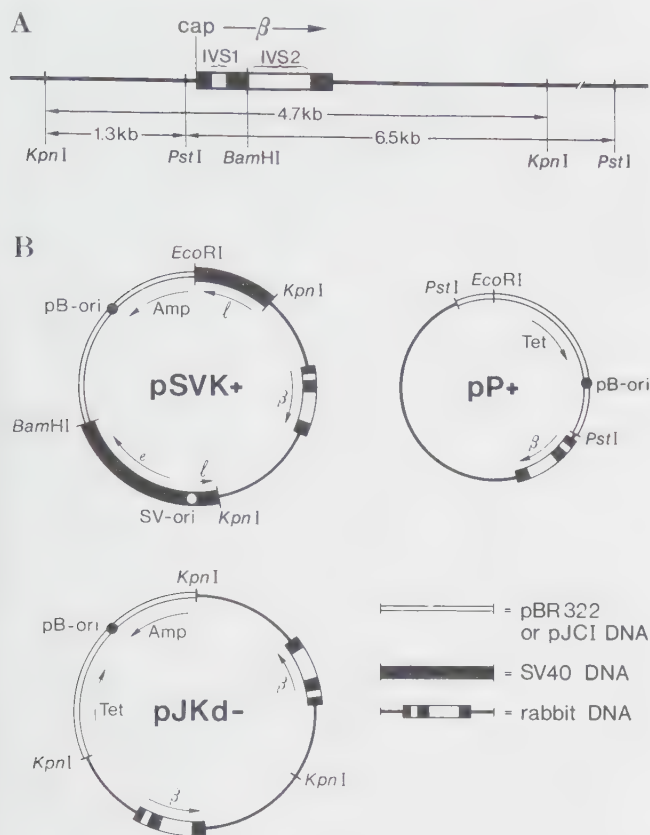


FIG. 1. (A) Schematic representation of the genomic rabbit hemoglobin β -1 gene (16, 18). The black line is noncoding DNA, the black bars are coding regions, and the open bars are intervening sequences (IVS1 and IVS2). (B) Recombinant DNAs containing the β -1 globin gene. Symbols not identified in the figure are: Tet, tetracycline resistance genes; Amp, β -lactamase gene; pB-ori and SV-ori, plasmid and SV40 origins of replication; e , SV40 early region; ℓ , late region.

through a 0.8% agarose gel and transferred to nitrocellulose as described in ref. 30. The transferred DNA was hybridized to a ^{32}P -labeled nick-translated probe of pBR322 recombinant plasmid containing the rabbit β -globin gene. S1 nuclease mapping of transcripts was done as described by Weaver and Weissmann (ref. 31; Fig. 4A). Splicing of the first intron was detected by using a *Pst* I/*Bam*HI restriction fragment (position -99 to +480; ref. 19) obtained from the genomic β -globin gene and ^{32}P -labeled at the *Bam*HI site as the hybridization probe. The probe DNA was denatured, hybridized to unlabeled RNA, and treated with S1 nuclease, and the products were analyzed by gel electrophoresis. Genuine rabbit β -globin mRNA (a gift of S. Nagata and C. Weissmann, Zürich) protected a labeled DNA fragment of 208 nucleotides spanning the region from the acceptor site of the first intron to the *Bam*HI site (position 272 to 480). In order to map the 5' end of the transcripts, a globin gene clone from which the first intron had been deleted (designated Z-pBR/Rc β G-A4, obtained from H. Weber, Zürich) was labeled at the *Bam*HI site and used as a hybridization probe. Unlabeled β -globin mRNA protected a DNA fragment of 354 nucleotides, from the cap site to the *Bam*HI site (position 1 to 480 of the genomic gene).

RESULTS

Toxicity of the Injected DNA. pBR322- β -globin recombinant DNA was injected into a total of 5239 fertilized eggs, either in a supercoiled form or after cutting at the single *Ava* I site within pBR322. In a large fraction of the embryos, development was not affected when up to 250 pg of DNA (about 2×10^7

genes) was injected per egg. Larger DNA quantities were increasingly toxic. With supercoiled DNA, 40%, 19%, 5%, and less than 1% of the embryos reached the swimming larva stage after injection of 0.25 ng, 0.5 ng, 1 ng, or 2 ng of supercoiled DNA, respectively. Linear DNA was found to be less toxic than supercoiled DNA (59%, 30%, 16%, and 7% swimming larvae after injection of 0.25 ng, 0.5 ng, 1 ng, or 2 ng of DNA, respectively).

Replication and Persistence of Injected DNA. Total embryonic DNA was extracted at various stages during development, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled rabbit β -globin recombinant plasmid DNA. It was found that supercoiled DNA was converted into a relaxed but covalently closed circular form immediately after injection. A few hours later, supercoiled DNA reappeared, as was found with circular DNA injected into frog oocytes (32). In addition, there was a large net increase of injected DNA sequences, reaching a maximal copy number at late gastrula (Fig. 2). The extent of DNA replication varied from one experiment to another, ranging from a 10- to 200-fold amplification of the input DNA, as determined by scintillation counting of membrane filter strips from Southern blots and dot hybridizations (e.g., egg 7 cpm, blastula 466 cpm, late gastrula 1480 cpm, feeding larva 76 cpm). We found that efficient replication depended on the amount of DNA injected. Whereas there was little replication with 125 pg of foreign DNA per egg (data not shown), extensive replication was observed with 250 pg or, in particular, 500 pg per egg (Fig. 2). Replication was not significantly different when the injected DNA was supercoiled or linear, or when it contained pBR322, an SV40 replicon, or both (Fig. 2; unpublished observations). As can be seen in Fig. 2A, globin gene sequences appeared in high molecular weight bands, but there was also a large increase of DNA migrating in

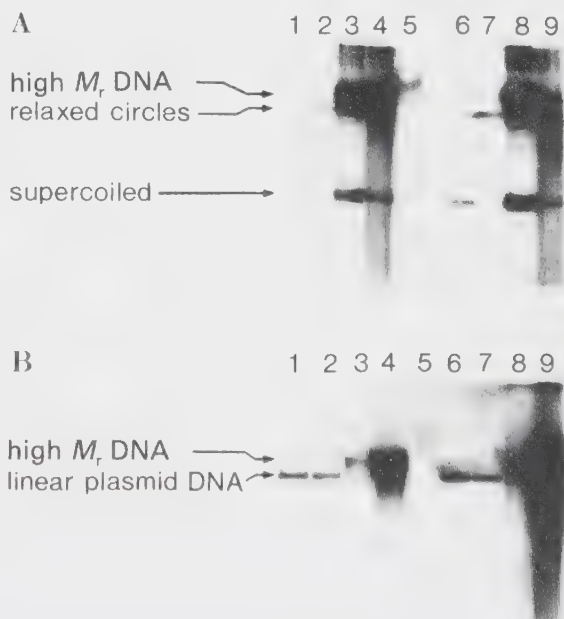


FIG. 2. Replication of injected rabbit β -globin genes in *Xenopus* embryos. DNA was extracted from injected embryos at various developmental stages, and the DNA equivalent of one embryo was electrophoresed through agarose gel, transferred to nitrocellulose, hybridized to ^{32}P -labeled globin gene probe, and autoradiographed. (A) Injection of supercoiled recombinant DNA (pP+). (B) Injection of linear recombinant DNA (pSVK+). Slots 1-5, 250 pg of DNA injected per egg; slots 6-9, 500 pg of DNA injected per egg. Slots 1 and 6, sample of DNA equivalent to amount injected; slots 2 and 7, DNA extracted from eggs 2 min after injection; slots 3 and 8, DNA from late blastula (stage 9); slots 4 and 9, DNA from late gastrula (stage 13); slot 5, DNA from swimming larvae (stages 41-45). M_r , molecular weight.

the position of supercoiled input plasmid DNA. The latter indicates that the injected DNA at this stage replicated autonomously rather than as part of *Xenopus* chromosomal DNA. After the gastrula stage, most of the foreign DNA was degraded. However, the remaining DNA was found to persist for many months. In gel electrophoresis, globin gene-pBR322 recombinant DNA migrated together with high molecular weight *Xenopus* DNA. Six-week-old tadpoles as well as 6-month-old frogs contained an average of 3–10 rabbit β -globin gene copies per cell, as judged from the intensity of the autoradiographic signals in Southern blots (Fig. 3). This calculation was based on a DNA content of 6 pg per diploid *Xenopus* cell (14) and a size of 13 kb for the pJKd- plasmid containing two globin genes.

DNA from tadpoles and frogs originally injected with supercoiled pJKd- plasmid was analyzed by restriction endonuclease digestion and Southern blotting (29, 30). We concluded that most of the persisting foreign DNA was present as head-to-tail tandem repeats of the original circular input DNA, because the persisting DNA was present in a high molecular weight form and yet gave the same restriction pattern as the input DNA. Digestion of the DNA with *Bst*EII, which does not cut the input pJKd- DNA, gave a hybridizing band of very high molecular weight (Fig. 3). The possibility that this high molecular weight band was the result of trapping of input plasmid DNA within high molecular weight *Xenopus* DNA was eliminated by the following reconstruction experiment: pJKd- plasmid DNA was mixed with *Xenopus* DNA, digested with *Bst*EII, and electrophoresed in parallel with tadpole DNA from injection experiments. The plasmid DNA migrated as supercoiled and relaxed circles and not with the high molecular weight DNA (Fig. 3). Digestion of the DNA with *Hind*III (Fig. 3), *Ava* I, or *Sal* I (data not shown), all cutting the input plasmid once but at different

positions, yielded a prominent band at the position of linear pJKd- DNA. Digestion with *Eco*RI and *Pst* I gave the same fragment pattern for both the persisting DNA and the input plasmid DNA (data not shown). So far, the findings are also compatible with the assumption that the foreign DNA persisted as catenanes (interlocked circles; ref. 33) of the circular input plasmid DNA. This seems unlikely because of digestion experiments with dilute DNase I: In a reconstruction experiment circular pJKd- DNA was mixed with *Xenopus* DNA and treated with increasing concentrations of DNase I. Southern blotting showed that the plasmid circles were nicked and then opened to linear form III DNA before they were degraded to smaller fragments. Under the same conditions, tandem multimers would be expected to yield a smear of random degradation products only. In fact, Southern blot analysis of DNA from tadpoles revealed that the persisting β -globin-pBR322 sequences were converted by DNase I from high molecular weight DNA to heterogeneous smaller fragments with no detectable intermediate of linear plasmid DNA (data not shown).

Correct and "Incorrect" Transcription of the Rabbit β -Globin Gene. Transcription of the injected rabbit β -globin gene was detected by the S1 nuclease mapping technique (31). DNA from the genomic clone was 32 P-labeled in the minus strand at the *Bam*HI site and hybridized to RNA extracted from the transformed *Xenopus* gastrulae (Fig. 4A). After S1 nuclease digestion, denaturation, and gel electrophoresis, a strong band, approximately 208 nucleotides in size, was found with RNA from late gastrulae (Fig. 4B, lanes 1 and 2). The same band appeared in reconstruction experiments with authentic rabbit globin mRNA (lanes 5–7) but was absent in control experiments using RNA from uninjected embryos (data not shown). A band of this size is best explained by correct splicing of the small intron in the globin gene transcript. Because 1 ng of β -globin mRNA corresponds to 3×10^9 molecules, we calculated that there were about 10^9 globin gene transcripts per embryo at gastrula stage. The protected 208-nucleotide DNA fragment was much weaker, but clearly identifiable in the autoradiograms, using RNA from hatching larvae (Fig. 4B, lanes 3 and 4). It appears as if the steady-state level of rabbit globin gene transcripts was roughly proportional to the number of gene copies, because hatching larvae contained much less of the injected DNA sequences than did late gastrulae (Fig. 2).

Using the same S1 nuclease mapping technique but a different probe (see *Materials and Methods*), we mapped the 5' ends of the transcripts (Fig. 4C). All of the clones containing pBR322 sequences gave rise to an incorrect 5' end, mapping approximately 306 nucleotides upstream from the *Bam*HI site (Fig. 4C, lanes 1, 2, and 4). However, further experiments using the circularized globin gene *Kpn* I fragment showed correct expression; the major protected DNA fragment was about 355 nucleotides long and comigrated with the fragment protected by genuine rabbit β -globin mRNA (Fig. 4C, lane 5). The band of full-length DNA probe present in all slots could be due to renaturation of the probe or the presence of transcripts that had initiated upstream of the *Pst* I site. Both the transcripts with the correct 5' ends and those with the incorrect 5' ends appeared to be polyadenylated, because they were selectively bound to an oligo(dT)/cellulose column (data not shown).

DISCUSSION

There have been several reports on the replication of foreign DNA injected into unfertilized eggs of *X. laevis* (7–12). Recently, Harland and Laskey (12) presented convincing evidence, based on density shift, for semiconservative replication of a variety of DNAs injected into unfertilized *Xenopus* eggs. In this paper we present evidence by Southern blotting for a large net increase of DNA injected into fertilized *Xenopus* eggs.



FIG. 3. Analysis of persisting rabbit β -globin genes in tadpoles and frogs. DNA was extracted from tadpoles and frogs, digested with restriction endonuclease, and analyzed as described in *Materials and Methods*, except that a 0.45% agarose gel was used in order to get high resolution of large molecular weight DNA fragments. One microgram of bacteriophage λ DNA was added to each DNA sample to test for complete digestion. (A) Gel stained with ethidium bromide. (B) Autoradiograph of the same gel after Southern blotting and hybridization to globin gene probe. Slots 1, 2, and 5, DNA from injection experiments with supercoiled pJKd- recombinant plasmid; slots 1 and 2, 4- μ g DNA samples from a pool of ten 6-week-old tadpoles; slot 5, 4- μ g DNA sample from a pool of three 6-month-old frogs; slots 3 and 4, reconstruction experiment in which 100 pg of pJKd- DNA was mixed with *Xenopus* DNA prior to digestion; slots 1, 3, and 5, restriction digestion with *Hind*III (cuts pJKd- DNA once); slots 2 and 4, digestion with *Bst*EII (does not cut pJKd-); slot M, bacteriophage λ marker DNA cut with *Eco*RI and *Hind*III.

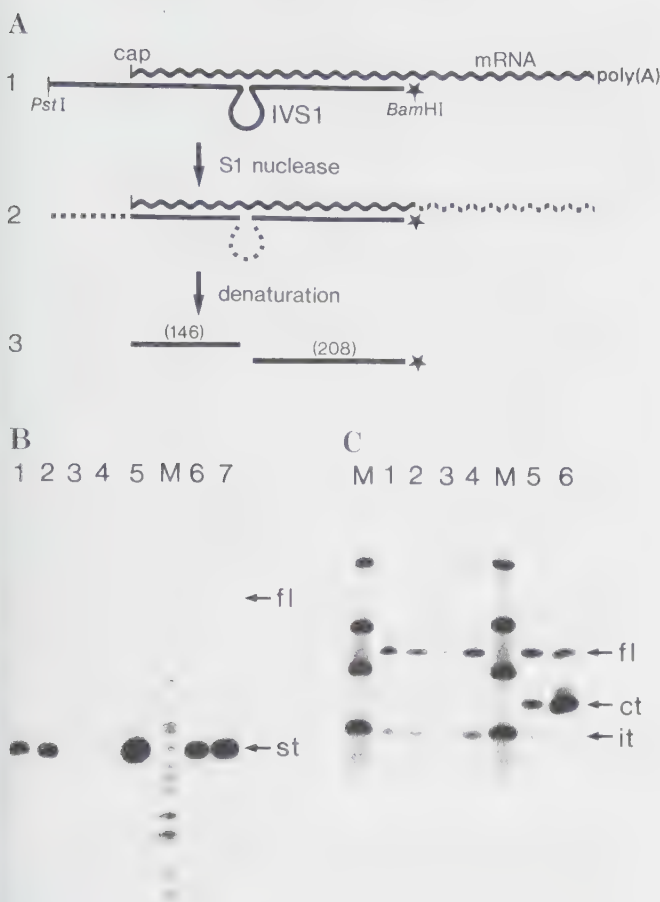


FIG. 4. Analysis of transcripts by S1 nuclease mapping. (A and B) Detection of correct splicing. End-labeled globin DNA was hybridized to unlabeled RNA from injected embryos, treated with S1 nuclease, denatured, fractionated by gel electrophoresis, and autoradiographed. The RNA was from pools of ten embryos; one-fifth was used for S1 nuclease mapping. Slots 1 and 2, hybridization to RNA from late blastulae (stage 13); slots 3 and 4, hybridization to RNA from hatching larvae (stage 35); slots 1 and 3, injection experiments with pSVK+ DNA; slots 2 and 4, injection experiment with pP+ DNA; slot 5, hybridization to total RNA from rabbit reticulocytes; slots 6 and 7, hybridization to 1 and 2.5 ng of purified rabbit β -globin mRNA, respectively; M, 32 P-labeled marker DNA fragments of pBR322 digested with *Hpa* II (22). fl, Full-length input DNA (579 nucleotides); st, splice terminus fragment (208 nucleotides). (C) Detection of the correct 5' terminus of globin RNA. For this experiment, a globin gene clone lacking the first intron (IVS1) was used as a radioactive probe. DNA end-labeled at the *Bam*HI site was hybridized to unlabeled RNA from gastrula stage embryos and treated as above. Slot 1, RNA from embryos injected with pJKd- DNA; slot 2, injected with pSVKd+ DNA; slot 3, RNA from uninjected embryos; slot 4, injected with pSVKd- recombinant DNA; slot 5, injected with plasmid-free globin gene *Kpn* I fragment dimer circles; slot 6, rabbit β -globin mRNA; M, 32 P-end-labeled marker DNA fragments of pBR322 digested with *Hpa* II. fl, Full-length input DNA (453 nucleotides); ct, fragment with correct terminus, mapping 146 + 208 = 354 nucleotides upstream of the *Bam*HI site; ic, incorrect terminus, about 306 nucleotides upstream of the *Bam*HI site.

We found that by 20 hr after injection the injected DNA was amplified 10 to 200 times. Linear DNA was readily ligated to large concatemeric DNA (which was also observed for other linear DNAs injected into unfertilized eggs; ref. 34; G. Galli, R. Clerc, and M. L. Birnstiel, personal communication) and was replicated in a high molecular weight form. Circular DNA seems to replicate as such, showing a considerable increase in intensity of the band of supercoiled DNA, together with the appearance of high molecular weight DNA forms (Fig. 2). It is

interesting to note that pCRI plasmid DNA and the circularized rabbit globin gene fragment both were replicated in developing *Xenopus* embryos (data not shown). These DNAs originally derive from prokaryotic and eukaryotic sources, respectively. It was also observed in our laboratory that circular or linear histone genes of a sea urchin were replicated in developing *Xenopus* embryos (35). Taken together, these findings could mean that on all the injected DNAs there was at least one functional replication origin or, as suggested by the data of Harland and Laskey (12), that there is no requirement for specific replication origins in *Xenopus* early development.

The fate of the injected DNA differed from that of the host embryonic *Xenopus* DNA. While the cell number increased from 1 to about 3×10^4 cells at gastrula stage, the foreign DNA increased 10- to 200-fold, and a large fraction of it was degraded between the late gastrula and the hatching larva stage. The fact that most of the foreign DNA was degraded after gastrula stage could mean that DNA replication in late embryos becomes dependent on specific replication origins, and that only those globin genes persisted that had been integrated into host cell DNA.

At later stages of development, the number of globin genes per cell appeared to be stable: Tadpoles containing an average of 3–10 rabbit globin genes per cell developed into healthy frogs with about the same number of rabbit genes per cell. During that period, the total number of cells had increased at least 10-fold. This means that the rabbit globin genes had become a stable component of the genome in a fraction or in all of the tadpole cells. The persisting DNA in older tadpoles had the same restriction map as the circular input DNA; however, it comigrated with high molecular weight *Xenopus* DNA prior to restriction digestion, or after digestion with *Bst*EII, which cuts *Xenopus* DNA but not the input recombinant plasmid. We conclude that the globin genes persisted as head-to-tail multimers of the original input plasmid DNA. Our results thus differ from the ones of Gordon *et al.* (36), who injected recombinant plasmid DNA into fertilized mouse eggs and found that grossly rearranged forms of the input DNA persisted in a small fraction of newborn animals. A head-to-tail multimer organization is natural for many repeated genes—e.g., rRNA genes, histone genes, and tRNA genes (reviewed in ref. 37). Head-to-tail duplications and multimers were also found for viral DNA in transformed cells (38–40). The generation and persistence of head-to-tail tandem multimers after injection of circular DNA is independent evidence for the replication of input DNA in frog embryos. Multimers could arise from “rolling circle” replication, like the amplified rRNA genes in amphibian oocytes (41, 42), or from multiple rounds of “Cairns mode” replication without segregation of daughter molecules, as had been reported for mitochondrial and viral DNAs (reviewed in ref. 43). The available data do not allow us to determine whether the β -globin gene repeats were themselves part of a chromosome or minichromosome, or if they were extrachromosomal linear or circular entities.

An important finding during the course of our investigations was that the rabbit β -globin gene is transcribed in developing frog embryos. Previously, Gurdon and Brown (14) reported that after injection of gradient-purified 5S RNA genes into fertilized *Xenopus* eggs, there was synthesis of RNA that hybridized back to the injected DNA. Using the refined analysis of transcripts by S1 nuclease mapping, we found that rabbit β -globin gene transcripts with the correct 5' end and splicing of at least one intron could be observed in *Xenopus* embryos. The correct 5' end, corresponding to the 5' terminus of rabbit β -globin mRNA, was found, however, only after injection of plasmid-free circular globin gene. Globin DNA linked to pBR322 (pP+, pJKd-), or to SV40 DNA in a pBR322-SV40 vector (pSVK+,

pSVKd+, pSVKd-) gave rise to transcripts with an incorrect 5' terminus. This occurred despite the fact that in pJKd-, pSVK+, and pSVKd- there were 1.4 kb of rabbit DNA upstream of the globin gene capsite. In mammalian cells transfected with rabbit β -1 globin genes from different sources (16, 20), the same incorrect terminus was also observed either as the only terminus (44) or together with transcripts containing correct 5' termini (ref. 45; unpublished data). This dependence of correct vs. incorrect transcription on neighboring sequences indicates a *cis*-acting effect of remote DNA sequences on gene expression (see also ref. 46). In late gastrulae (Daudin stage 13) there were 10^9 rabbit globin gene copies per embryo, which is an average of about 3×10^4 genes per cell. At the same time S1 nuclease mapping experiments indicated that there were about 10^9 molecules of rabbit globin gene transcripts per embryo. The low ratio of steady-state β -globin transcripts to β -globin genes (1:1) could be due to a reduced rate of transcription, the inability of all globin genes to be utilized as templates, or instability of the globin gene transcripts. Nevertheless, the rabbit globin gene transcripts must have represented about 1.4% of the poly(A)⁺ RNA molecules of transformed frog embryos (47). This amount exceeds the high levels observed for several mRNA species transcribed from endogenous *Xenopus* genes (48).

It will be of great interest to see whether in tadpoles and frogs there will be tissue-specific expression of injected heterologous or homologous genes that normally are under developmental control.

We are indebted to Drs. T. Maniatis (Pasadena), H. Weber and F. Meyer (Zürich), S. Nagata and C. Weissmann (Zürich), and J. Jenkins (London) for gifts of genomic globin clone, globin gene plasmids, globin mRNA and pJC-1 plasmid, respectively. We are grateful to R. Weiss, P. Wettstein, F. Ochsenbein, and S. Oberholzer for preparing plasmid DNA, hormone-induced frogs, graphic figures, and the typed manuscript, respectively. We gratefully acknowledge R. Clerc's instruction for fertilizing eggs *in vitro*. We also thank Prof. M. L. Birnstiel for support and for valuable discussions, and Drs. M. Bendig, P. Dierks, and M. Botchan for critical reading of the manuscript. This work was supported by the Schweizerische Nationalfonds, Grants 3.337.0.78 and 3.687.0.80, and by the Kanton Zürich.

1. Lane, C. D. & Knowland, J. (1975) in *The Biochemistry of Animal Development*, ed. Weber, R. (Academic, New York), Vol. 3, pp. 145-181.
2. Kressmann, A., Clarkson, S. G., Telford, J. L. & Birnstiel, M. L. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 1077-1082.
3. Laskey, R. A., Honda, B. M., Mills, A. D., Morris, N. R., Wyllie, A. H., Mertz, J. E., De Robertis, E. M. & Gurdon, J. B. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 171-178.
4. Cortese, R., Melton, D., Tranquilla, T. & Smith, J. D. (1978) *Nucleic Acids Res.* **5**, 4593-4611.
5. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1432-1436.
6. Birnstiel, M. L. & Chipchase, M. (1977) *Trends Biochem. Sci.* **2**, 149-152.
7. Gurdon, J. B., Birnstiel, M. L. & Speight, V. A. (1969) *Biochim. Biophys. Acta* **174**, 614-628.
8. Laskey, R. A. & Gurdon, J. B. (1973) *Eur. J. Biochem.* **37**, 467-471.
9. Ford, C. C. & Woodland, H. R. (1975) *Dev. Biol.* **43**, 484-491.
10. Benbow, R. M., Krauss, M. R. & Reeder, R. H. (1978) *Cell* **13**, 307-318.
11. Watanabe, S. & Taylor, J. H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5292-5296.

12. Harland, R. M. & Laskey, R. A. (1980) *Cell* **21**, 761-771.
13. Gurdon, J. B. (1974) *Nature (London)* **248**, 772-776.
14. Gurdon, J. B. & Brown, D. D. (1977) in *The Molecular Biology of the Mammalian Gene Apparatus*, ed. Ts'o, P.O.P. (Elsevier/North Holland, Amsterdam), pp. 111-123.
15. Gurdon, J. B., Woodland, H. R. & Lingrel, J. B. (1974) *Dev. Biol.* **39**, 125-133.
16. Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. & Efstratiadis, A. (1978) *Cell* **15**, 687-701.
17. Maniatis, T., Sim, G. K., Efstratiadis, A. & Kafatos, F. C. (1976) *Cell* **8**, 163-182.
18. Hardison, R. C., Butler, E. T., III, Lacy, E., Maniatis, T., Rosenthal, N. & Efstratiadis, A. (1979) *Cell* **18**, 1285-1297.
19. van Ooyen, A., van den Berg, J., Mantei, N. & Weissmann, C. (1979) *Science* **206**, 337-344.
20. Van den Berg, J., Van Ooyen, A., Mantei, N., Schamböck, A., Grosveld, G., Flavell, R. A. & Weissmann, C. (1978) *Nature (London)* **276**, 37-44.
21. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977) *Gene* **2**, 95-113.
22. Sutcliffe, J. G. (1979) *Nucleic Acids Res.* **5**, 2721-2728.
23. Schaffner, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2163-2167.
24. Probst, E., Kressmann, A. & Birnstiel, M. L. (1979) *J. Mol. Biol.* **135**, 709-732.
25. Morrow, J. F., Cohen, S. N., Chang, A. C. Y., Boyer, H. W., Goodman, H. M. & Helling, R. B. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3455-3459.
26. Grunstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961-3965.
27. Billett, F. S. & Wild, A. E. (1975) in *Practical Studies of Animal Development*, (Chapman & Hall, London), pp. 119-127.
28. Nieuwkoop, P. D. & Faber, J. (1967) in *Normal Table of Xenopus laevis (Daudin)* (North-Holland, Amsterdam).
29. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503-517.
30. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683-3687.
31. Weaver, R. & Weissmann, C. (1979) *Nucleic Acids Res.* **7**, 1175-1193.
32. Wyllie, A. H., Laskey, R. A., Finch, J. & Gurdon, J. B. (1978) *Dev. Biol.* **64**, 178-188.
33. Baldi, M. I., Benedetti, P., Mattochia, E. & Tocchini-Valentini, G. P. (1980) *Cell* **20**, 461-467.
34. Clerc, R. (1980) *Dissertation* (Univ. Zürich, Zürich, Switzerland).
35. Bendig, M. M. (1981) *Nature (London)*, **292**, 65-67.
36. Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A. & Ruddle, F. H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7380-7384.
37. Long, E. D. & Dawid, I. B. (1980) *Annu. Rev. Biochem.* **49**, 727-764.
38. Botchan, M., Topp, W. & Sambrook, J. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 709-719.
39. Schaffner, W., Topp, W. & Botchan, M. (1979) in *Specific Eukaryotic Genes, Alfred Benzon Symp.*, eds. Engberg, J., Klenow, H. & Leick, V. (Munksgaard, Copenhagen), Vol. 13, pp. 191-203.
40. Birg, F., Dulbecco, R., Fried, M. & Kamen, R. (1979) *J. Virol.* **29**, 633-648.
41. Hourcade, D., Dressler, D. & Wolfson, J. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2926-2930.
42. Rochaix, J. D., Bird, A. & Bakken, A. (1974) *J. Mol. Biol.* **87**, 473-487.
43. Kasamatsu, H. & Vinograd, J. (1974) *Annu. Rev. Biochem.* **43**, 695-719.
44. Wold, B., Wigler, M., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5684-5688.
45. Dierks, P., van Ooyen, A., Mantei, N. & Weissmann, C. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1411-1415.
46. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7102-7106.
47. Sagata, N., Shiokawa, K. & Yamana, K. (1980) *Dev. Biol.* **77**, 431-448.
48. Perlman, S. & Rosbash, M. (1978) *Dev. Biol.* **63**, 197-212.

ARBEIT Nr. 2

Cloned Genes Injected into Xenopus Zygotes
Give Rise to Genetically Mosaic Frogs.

Sandro Rusconi and Walter Schaffner
Institut für Molekularbiologie II
der Universität Zürich
Hönggerberg, 8093 Zürich, Switzerland

Telephone: (01) 377' 34 48

ABSTRACT

Our earlier studies (Rusconi & Schaffner, 1981) demonstrated that Xenopus laevis embryos can be successfully transformed by microinjection of rabbit β -globin gene DNA at the zygote stage. The injected DNA is amplified extrachromosomally at early stages of development and is degraded after gastrulation occurs. However, a minor fraction of the foreign genes is more stable and persists, probably integrated into the host genome, in the form of tandem oligomers. Closer analysis of DNA from transformed frogs shows that the persisting heterologous sequences are unevenly distributed among different tissues of the same individuals, without any apparent tissue preference. Moreover, the heterologous sequences remain hypomethylated after several months of propagation in the eukaryotic environment. Nevertheless, none of the rabbit β -globin genes are transcribed at a detectable level in the frog tissues, whereas our earlier studies showed them to be correctly transcribed at gastrula stage when the majority of injected DNA was present in an extrachromosomal state.

INTRODUCTION

Oocytes and eggs of Xenopus laevis are valuable tools for testing the activity of biological macromolecules. Oocytes have been injected with protein (reviewed by Lane, 1981), RNA (Woodland et al., 1974; Woodland & Wilt, 1980a), and DNA (reviewed by Gurdon & Melton, 1981) molecules. The unfertilized egg has been particularly useful in studying the replication of injected DNA (Harland & Laskey, 1980; Hines & Benbow, 1982). mRNA (Woodland & Wilt, 1980b; Gurdon et al., 1984) and genomic DNA

(Gurdon & Brown, 1977) injected into fertilized eggs is maintained in active state for a while but seems to disappear in later developmental stages. Only with the advent of DNA cloning techniques has become possible to inject defined protein-coding genes into the fertilized frog egg and to study their fate throughout development. To this end, cloned genes coding for the rabbit β -globin gene (Rusconi & Schaffner, 1981) and for sea urchin histone genes (Bendig, 1981) were used in our laboratory. Both kind of genes were replicated as extrachromosomal circles within developing *Xenopus* embryos and were correctly transcribed around blastula-gastrula stage. Whereas most injected DNA was degraded after gastrula stage, a low number of rabbit β -globin genes became stably associated with the Xenopus genome and persisted throughout metamorphosis. In the present paper we take a closer look at the state of such injected DNA sequences.

MATERIALS AND METHODS

Microinjection of Xenopus laevis fertilized eggs and isolation of nucleic acids at different developmental stages have been previously described (Rusconi & Schaffner, 1981). For the analysis shown in Fig.1, undigested material was used whereas, in the other cases, the DNA (usually 3 μ g) was digested with various restriction enzymes as indicated in the figure legends. Incubation with HpaII and MspI was performed under conditions of 20-fold overdigestion (5 x enzyme excess and 4 x incubation time). Other enzymes were used as recommended by the manufacturers (Boehringer Mannheim and New England Biolabs). If two successive digestions were required on the same sample, they were separated by phenol-chloroform extraction and ethanol

precipitation. Agarose gel electrophoresis and Southern blot analysis were carried out as described (Rusconi & Schaffner, 1981). Dot blot analysis was performed essentially according to the protocol of Kafatos et al. (1979).

RESULTS AND DISCUSSION

Fate of the DNA after injection

In a typical experiment, about 10^7 DNA molecules were injected into each fertilized egg. One might have expected that the majority of the transferred molecules would not integrate into the host genome and would progressively disappear. However, in very early stages of development (from 0 to about 24 hrs) every DNA tested so far (including pure prokaryotic vectors such as pBR322 plasmid DNA) is able to undergo autonomous replication. In many cases this replication is so efficient that the newly synthesized DNA forms a visible band in agarose gel electrophoresis of undigested material from single embryos. Fig.1a shows an example of such DNA bands which appear in undigested material extracted from blastula or gastrula (see lanes 2 and 3) but which cannot be detected in material extracted immediately after injection of 250 pg of recombinant DNA (lane 1). As mentioned above, this ability to replicate is a general property of all the DNA molecules tested so far. Harland and Laskey (1980) suggested that during early developmental stages of X.laevis there may be no requirement for specific origins of replication. For example they showed that each one of the six circularized HindIII fragments of simian virus 40 DNA is replicated after injection into activated X.laevis eggs. However, this still does not exclude a preference for sequences related to

specific origins of replication (Harland & Laskey, 1982). During early embryogenesis frog DNA replicates at a very high rate (two to three times every hour). Use of sequences related to specific origins of replication might facilitate recruitment of a large number of sites for initiation of replication. At the beginning of gastrulation, when the frequency of cell divisions decreases, a more stringent mode of replication would again prevail. Consistent with this hypothesis we observe that all the extrachromosomal recombinant DNA which may have accumulated because of a transient high permissivity for replication gradually disappears after gastrulation. After about 30 days of development, the amount of foreign DNA increases in parallel with genomic DNA indicating that the two DNAs replicate at the same rate (see Fig.1c).

We also studied the tissue distribution of injected DNA at later developmental stages. For this purpose DNA was extracted from different tissues of 5-6 month-old frogs which had been injected at the zygote stage with rabbit β -globin gene DNA. Only three out of ten analyzed individuals lacked detectable rabbit sequences. In two other individuals the foreign genes were present at low copy number (less than one copy per cell) in at least one of the organs examined. The remaining individuals (50%) contained higher copy numbers (from two to fifty copies per cell) unevenly distributed among the tissues. These observations are in good agreement with the previously reported values of three to ten copies per cell (Rusconi and Schaffner, 1981) obtained with pools of frogs. Fig.2a shows four Southern blot analyses of PstI-digested DNA from blood (B), leg muscle (M), liver (L) and the remaining parts of the body (R). Every lane contains the same

amount of DNA so that the number of persisting gene copies can be directly estimated by comparison with known standards (shown in Fig.2: 1 and 50 copies per cell). The intensity of hybridization is subject to extensive variation within the same organism. The analyzed tissues or organs are also equally permissive for the presence of the heterologous sequences. For example, the rabbit globin DNA is highly represented in the blood of frog No.11 but is absent in the blood of other frogs. We therefore conclude that the transformed frogs were mosaics for the heterologous gene. Integration into the host genome at the one-cell stage would have resulted in homologous distribution of the foreign sequences in the frogs. Therefore, multiple integration events probably occurred throughout the cleavage stages when the injected molecules were maintained at a high concentration due to extrachromosomal replication. Upon digestion with various restriction enzymes (see PstI and HpaII in figures 2 and 3, respectively) the integrated DNA releases the same bands as input circular DNA. There are no cases where a particular restriction fragment is missing or new hybridizing bands are present in equimolar amount to the expected ones. Additional faint bands are visible on top of a widely spread smear in the background. As suggested before (Rusconi & Schaffner, 1981) these data can be explained by assuming the existence of tandem head-to-tail oligomer inserts whose internal restriction pattern would correspond to that of circular DNA. The fragments generated at the junctions with host DNA would therefore have a different molecular weight and would generate additional sub-molar bands. A number of such junctions would create a variety of fragments of different size and could be responsible for the observed "smear"

hybridization. According to our explanation, faint bands on top of the smear reflect some early integration events which have been clonally propagated to a larger extent in the analyzed compartment. The features mentioned above are not unique to transformation experiments with frogs. In addition to multiple, seemingly random integration events, the organization of the foreign DNA as head-to-tail oligomers has also been observed in gene transfer experiments with mammalian cells and organisms (Costantini & Lacy, 1981; Brinster et al., 1981; Perucho et al., 1980; Robins et al., 1981).

Expression of the foreign genes

In our previous work on frog transformation we noted that the injected rabbit β -globin genes were specifically transcribed during early phases of development (Rusconi & Schaffner, 1981). S1-nuclease mapping of the transcripts showed that most of them were initiated at the same point as in rabbit reticulocytes. The amount of steady-state transcripts was approximately equimolar to the number of gene copies present in the early embryo, reaching a maximal value at gastrula stage and decreasing as degradation of the heterologous episomes progressed.

Analysis at later stages of development failed to reveal any rabbit β -globin specific transcripts in tadpoles older than three weeks (data not shown). Even in frog tissues with relatively high copy numbers of foreign genes (for example the blood of frog No.11 with about 50 copies per cell), the amount of specific heterologous transcripts was below the detection level (i.e. less than 1 transcript per cell). Taken together, these observations suggest that expression of the heterologous globin gene is

restricted to the period in which the injected genes persist in episomal state. It seems as if integration into the host genome reduces or abolishes further transcription of the foreign gene.

The conversion of the persisting rabbit genes into such a "silent" state could have been due to de novo DNA methylation, since methylation has been repeatedly correlated with the inactive state of several vertebrate genes (reviewed by Felsenfeld & McGhee, 1982). The degree of methylation of some DNA sequences can be inferred from the digestion pattern obtained with certain restriction endonucleases (Razin & Riggs, 1980). A widely used system is the combined analysis with HpaII and MspI. Both enzymes recognize and cut the unmodified sequence 5'-CCGG-3' but are inactive if both C residues are methylated. Furthermore, HpaII does not cleave the site when the central C is methylated, whereas MspI does not cleave if the site is modified at the 5' terminal C. The methylation of the central C in CCGG is the most common in eukaryotes (the so-called CpG methylation), whereas the other two modifications are less frequent. Hence, MspI cleaves most of the CCGG sequences in "normally" methylated eukaryotic DNA (Razin & Riggs, 1980).

DNA from common cloning sources (in our case the E.coli strain HB101) is readily cut to completion with HpaII. Any modification of the HpaII susceptibility after DNA transfer into a eukaryotic environment will most likely be due to de novo methylation (Jähner et al., 1982). To test for such de novo methylation of the rabbit DNA in transformed frogs, we analyzed the HpaII- and MspI-digested material from several tissues by Southern blot. Fig.3 shows the analysis carried out with frog No.4. As previously shown (see Fig.2a), this individual contained

high copy numbers of the injected recombinant DNA in some of its organs. From the ethidium bromide staining shown in Fig.3 (lanes 1-4) it is evident that the bulk genomic DNA is resistant to HpaII cleavage (lanes 1 and 2) but can be almost completely digested with MspI (lanes 3 and 4). The bulk genomic DNA is, therefore, highly CpG methylated. A reconstruction experiment is shown (lanes 1, 3, 5 and 7) in which trace amounts of the injected DNA were mixed with genomic DNA extracted from untransformed frogs. As expected, the hybridization patterns reveal that the added control DNA is equally well digested by both HpaII and MspI (lanes 5 and 7). Unlike the bulk of X.laevis DNA the rabbit sequences persisting in the frog tissues are digested to essentially the same extent by both enzymes (lanes 6 and 8). Combined digestion experiments were also done by using HpaII and MspI together with enzymes insensitive to CpG methylation, like HindII and Taq I. We were particularly interested in the HpaII site located in the large intron of the rabbit β -globin gene, since its methylation pattern in the rabbit is tissue specific (Waalwijk & Flavell, 1978). As expected, digestion of the DNA of frog Nr.4 with Hind II releases a hybridizing fragment of 1.3 kb (arrow in panel c of Fig.3), which contains the HpaII site of interest. As shown in lane 10 of the same figure, subsequent HpaII digestion quantitatively converts this fragment to the smaller products (0.7 + 0.6 kb), indicating that this HpaII site persists in an unmodified state. Similar results were obtained with combined Taq I/HpaII digestion (Fig.3c, lanes 12-14), with other methylation-sensitive enzymes such as Sal I, Ava I and Xho I (not shown), and also with DNA from other transformed frogs (data not shown). Therefore we

conclude that de novo methylation of these sites is negligible, which probably means that most of the persisting heterologous sequences remain unmethylated at these CpG positions. It is not so surprising that de novo methylation does not occur in activated eggs during the first two replication cycles of injected DNA (Harland, 1982) but it is remarkable that there still is no methylation of the injected sequences after several months. Genes integrated into the host genomes of mouse eggs or early embryos become de novo methylated and at the same time inactivated, whereas integration into post-implantation embryos does not result in de novo methylation (Jähner et al., 1982). As mentioned, in our frog transformations integration events seem to occur late in embryonic development and this may be related to the absence of de novo methylation. Compared to transformation of mouse embryos at the post-implantation stage, our Xenopus transformation system offers an easy way of introducing and propagating unmethylated DNA in a vertebrate organism. However, it remains to be seen whether or not the correlation between DNA methylation and gene activation also applies to vertebrates other than mammals and birds. Whatever the role of DNA methylation may be for Xenopus, it is obvious that de novo methylation cannot explain the inactivity of the rabbit β -globin gene in transformed frogs. Our results are also compatible with the observation of MacLeod & Bird (1982) who reported that hypomethylation of certain sites on X.borealis rRNA genes is not sufficient to ensure their activation in X.laevis-borealis hybrid tadpoles.

There is a further possible explanation for conversion of the exogeneous gene into a quiescent state. For example, boundaries between euchromatin and heterochromatic regions of eukaryotic chromosomes seem to be preferred sites for sister chromatid exchange (Latt, 1981) and mitotic crossover (Tartof, 1975). One could argue that in most cases the foreign sequences are trapped by the host genome during these crossover/exchange events and therefore end up near to, or within highly packaged heterochromatin. These flanking regions could exert an unpredictable cis-effect, in a fashion analogous to the phenomena of position-variegation (Baker, 1968). Therefore it may be useful to develop techniques whereby the exogenous gene is directed into its homologous chromosomal locus, or maintained in an episome-like state. In our case it would be interesting to see whether passage through the germline to the offspring may allow the clonal propagation of simpler integration patterns and whether the newly acquired genes, previously devoid of CpG methylation, would then have a methylation pattern more similar to their natural counterparts. The handicap of the relatively long generation time of X.laevis, about one year under optimal conditions, would be partly compensated by the very high fertility of the frogs. Furthermore, one could clone single nuclei from transformed early embryos by transplanting them into enucleated eggs (Gurdon, 1977) as an alternative to the germline passage.

ACKNOWLEDGEMENTS

We thank Dr. T. Maniatis (Cambridge, Ma.) for the gift of the genomic rabbit globin gene. We are also grateful to Dr. M.L. Birnstiel for valuable discussions and to Dr. T. Sneider (Fort Collins, Co.) for critical reading of the manuscript. This work has been supported by the Schweiz. Nationalfonds, grant No.3.687.80 and by the Kanton Zürich.

REFERENCES

- BAKER, W.K. (1968). Position-effect variegation. *Adv.Genet.* 14, 133-169.
- BRINSTER, R.L., CHEN, H.I., TRUMBAUER, M., SENEAR, A.W., WARREN, R. and PALMITER, R.D. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27, 223-231.
- COSTANTINI, F. and LACY, E. (1981). Introduction of a rabbit β -globin gene into the mouse germ line. *Nature* 294, 92-94.
- FELSENFELD, G. and MC GHEE, J. (1982). Methylation and gene control. *Nature* 296, 602-603.
- GRUENBAUM, Y., CEDAR, H. and RAZIN, A. (1981). Restriction enzyme digestion of hemimethylated DNA. *Nucl.Acids Res.* 9, 2509-2415.
- GURDON, J.B. (1977). Methods for nuclear transplantation in amphibia. *Methods in Cell Biology* 16, 125-129.

- GURDON, G.B. and MELTON, D.A. (1981) Gene transfer in amphibian eggs and oocytes. *Ann.Rev.Genet.* 15, 180-218.
- GURDON, G.B., WOODLAND, H.R. and LINGREL, J.B. (1974). The translation of mammalian globin mRNA injected into fertilized eggs of *Xenopus laevis*. *Develop.Biol.* 39, 125-133.
- GURDON, G.B. and BROWN, D.D. (1977). Towards an in vivo assay for the function of nuclear macromolecules. In: *The Molecular Biology of the Mammalian Genetic Apparatus*, ed. P. T'so. pp.111-123. Amsterdam: North-Holland.
- HARLAND, R. (1982). Inheritance of DNA methylation in microinjected eggs of *Xenopus laevis*. *Proc.Natl.Acad.Sci.USA* 79, 2323-2327.
- HARLAND, R.M. and LASKEY, R.A. (1980). Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. *Cell* 21, 761-771.
- HARLAND, R.M. and LASKEY, R.A. (1982). Replication origins in the *Xenopus* egg. *Cell* 31, 503.
- HINES, P.J. and BENBOW, R.M. (1982). Initiation of replication at specific origins in DNA molecules injected into unfertilized eggs of the frog *Xenopus laevis*. *Cell* 30, 459-468.
- JAEHNER, D., STUHLMANN, H., STEWARD, C.L., HARBERS, K., LOEHLER, J., SIMON, I. and JAENISCH, R. (1982). De novo methylation and expression of retroviral genomes during mouse embryogenesis. *Nature* 298, 623-628.
- KAFATOS, F.D., JONES, C.W. and EFSTRATIADIS, A. (1979). Determination of nucleic acid sequence homologies and relative concentration by a dot blot hybridization procedure. *Nucl.Acids Res.* 7, 1541-1552.
- LANE, C.D. (1981). The fate of foreign proteins introduced into *Xenopus* oocytes. *Cell* 24, 281-282.

LATT, S.A. (1981). Sister chromatid exchange formation. *Ann.Rev. Genetic* 15, 11-55.

MACLEOD, D. and BIRD, A. (1982). DNAase I sensitivity and methylation of active versus inactive rRNA genes in *Xenopus* species hybrids. *Cell* 29, 211-218.

PERUCHO, M., HANAHAN, D. and WIGLER, M. (1980). Genetic and physical linkage of exogenous sequences in transformed cells. *Cell* 22, 309-317.

RAZIN, A. and RIGGS, A. (1980). DNA methylation and gene function. *Science* 210, 604-610.

ROBINS, D.M., RYPLEY, S., HENDERSON, H.S. and AXEL, R. (1981). Transforming DNA integrates into the host chromosomes. *Cell* 23, 29-39.

RUSCONI, S. and SCHAFFNER, W. (1981). Transformation of frog embryos with a rabbit β -globin gene. *Proc.Natl.Acad.Sci.USA* 78, 5051-5055.

TARTOF, K.D. (1975) Redundant genes. *Ann.Rev.Genet.* 9, 355-385.

WAALWIJK, C. and FLAVELL, R.A. (1978). DNA methylation at a CCGG sequence in the large intron of the rabbit β -globin gene: Tissue specific variations...

WOODLAND, H.R. and WILT, R.H. (1980a). The functional stability of sea urchin histone mRNA injected into oocytes of *Xenopus laevis*. *Dev.Biol.* 75, 199-213.

WOODLAND, H.R. and WILT, F.H. (1980b). The stability and translation of sea urchin histone mRNA molecules injected into *Xenopus laevis* eggs and developing embryos. *Dev.Biol.* 75, 214-221.

FIGURE 1 Fate of the injected DNA during development

a) Undigested material (1 embryo equivalent per slot) was separated by gel electrophoresis in 0.8% agarose and 1 mg/l ethidium bromide. Lane 1, nucleic acids extracted immediately after injection; lanes 2 and 3, material from blastula and gastrula stage, respectively. The arrow indicates the position of the supercoiled form of the injected β -globin gene recombinant plasmid (pJKd-; see Rusconi & Schaffner, 1981).

b) The samples shown in (a) after Southern transfer and hybridization with a ^{32}P -labelled probe homologous to the injected DNA. Lanes 4,5 and 6 of the resulting autoradiograph correspond to lanes 1,2 and 3 of (a), respectively.

c) Double logarithmic plot representing the amount of foreign DNA (symbols in the shaded area) and host genomic DNA ($\blacktriangle$) For various stages of development (E, egg; B, blastula; G, gastrula; S, swimming larva; L, feeding larva; T, 3 week-old tadpole; F, 5 month-old frog). The amount of foreign DNA molecules was estimated by Southern blots ($\blacksquare$) or dot blots (O). The data have been collected from several independent experimental batches, where different DNA clones were injected.

FIGURE 2 Mosaic distribution of the foreign sequences in young frogs

DNA was extracted from blood (B), leg muscle (M), liver (L), and the rest of the body (R), using the same procedure as for entire embryos. For the Southern blots shown here, 3 μg of PstI-digested DNA were loaded in each lane.

- a) Southern blot of DNA extracted from tissues of different individuals (No.11, No.7, No.5 and No.4). The two separate lanes at the left side show a reconstruction experiment (cloned rabbit β -globin DNA mixed with excess frog DNA) representing the intensities of hybridization expected when 1, respectively 50 gene copies per cell are present. The digestions of blood DNA are incomplete.
- b) Restriction map of the DNA used for most of the injections described in this article. The DNA is a dimer of the genomic 4.7 kb KpnI(K) fragment whose middle KpnI site (indicated as K*) has been destroyed. The circularized product obtained after incubation with T4 ligase was purified on agarose gel prior to injection. The PstI digest of such circularized dimers yields two globin gene fragments of different size, due to a short deletion which occurred during the elimination of the intervening KpnI site by S1 nuclease. Black bars and open bars represent exons and intervening sequences of the rabbit globin gene, respectively. Arrow, direction of transcription.

FIGURE 3 Absence of methylation in the persisting sequences

For the reconstruction experiment (lanes 1,3,5 and 7) a large excess of DNA from untransformed frogs was mixed with trace amounts (equivalent to about 70 copies per cell) of cloned DNA of the type shown in Figure 2b. The remaining lanes represent the analysis of DNA from frog No.4 (sample R, see Fig.2a).

- a) Ethidium bromide staining of the gel prior to transfer onto nitrocellulose. Lanes: 1 and 2, HpaII digestion; 3 and 4, MspI digestion. M, molecular weight standard (lambda phage DNA digested with EcoRI and HindIII).

b) Lanes 5-8, autoradiographs showing the hybridization pattern obtained from Southern blots of lanes 1-4. Lanes 6 and 8 are shown after a 3x longer exposure time in order to facilitate the comparison of the hybridization patterns.

c) 9 μ g of DNA from frog No.4, sample R, were digested with HindII. One third was used for lane 9, the rest was divided into two aliquots and further digested with HpaII (lane 10) or MspI (lane 11). The arrow indicates the position of the 1.3 kb HindII fragment harboring the HpaII site mentioned in the text.

d) A protocol analogous to the one described in (c) was followed for the combined digestion of DNA from frog No.4 with TaqI (lane 12), TaqI + HpaII (lane 13), and TaqI + MspI (lane 14). Arrow as in (c).

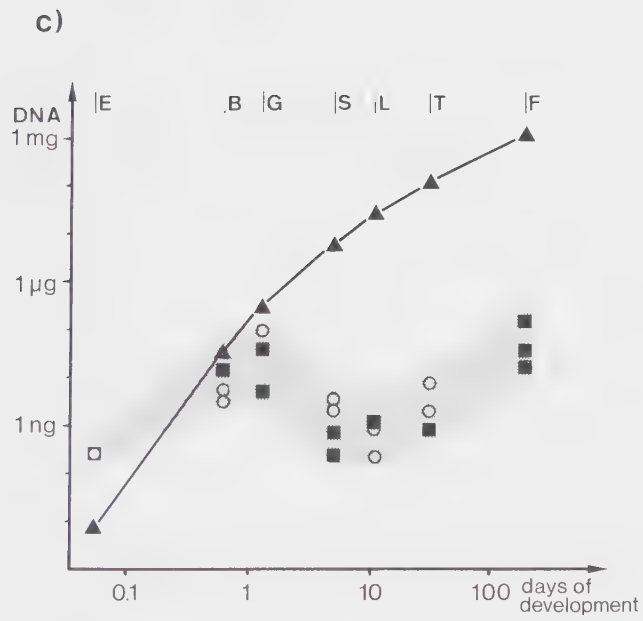
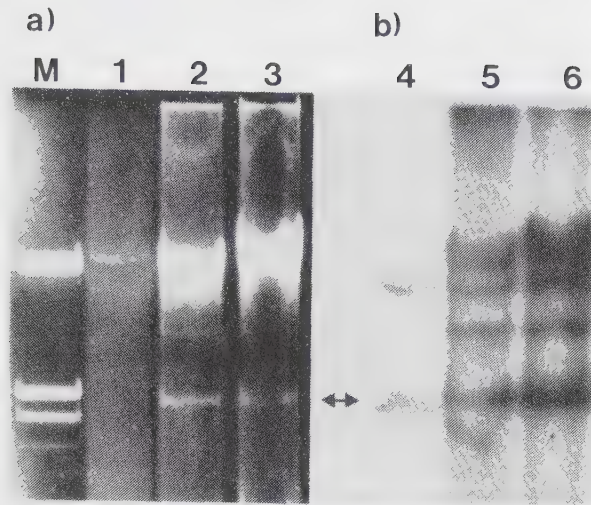


FIGURE 1

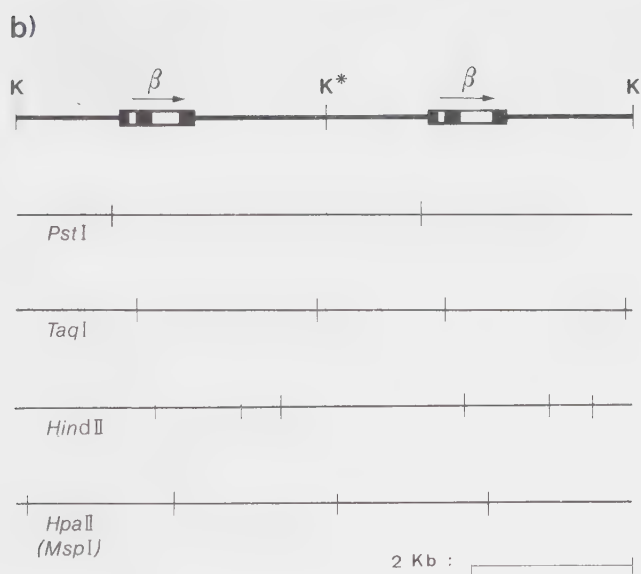
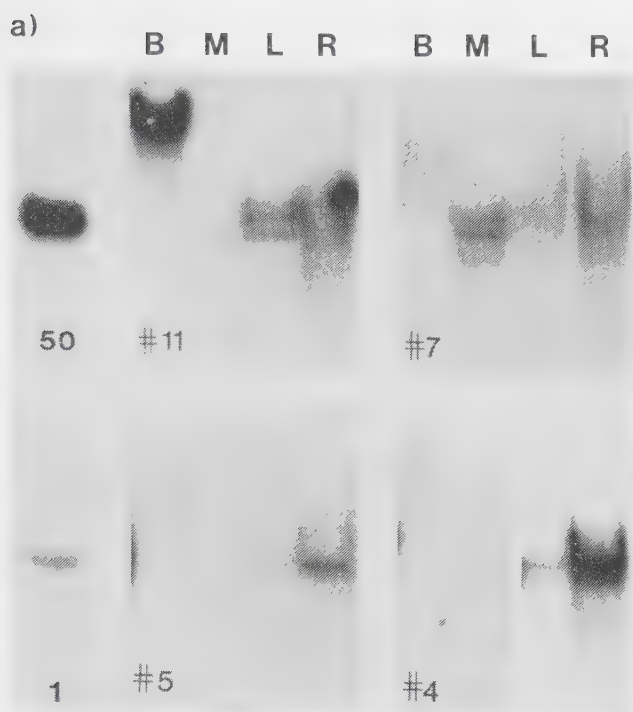


FIGURE 2

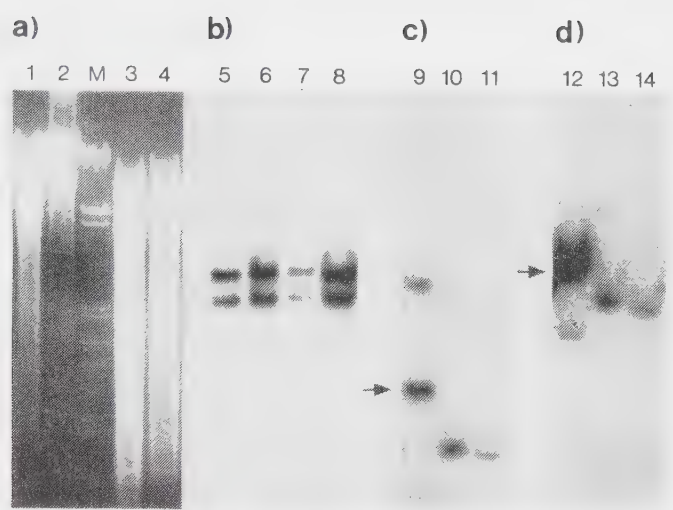


FIGURE 3

ARBEIT Nr. 3

Transformation of mice with an in vitro mutated
mouse α -globin gene.

Sandro Rusconi and Walter Schaffner
Institut für Molekularbiologie II
der Universität Zürich

Hönggerberg 8093 Zürich, Switzerland

(Telephone: (01) 377 34 48)

ABSTRACT

In order to allow the detection of a newly introduced homologous gene in a transgenic organism a strategy was devised whereby a cloned mouse α -globin gene was mutated in vitro by insertion of linker DNA in its 3' untranslated region. The mutated versions are shown to be expressed at a level comparable to the unmodified gene in a transient expression assay in HeLa cells. Furthermore, we show that the artificially introduced mutations are sufficient for the easy identification of (a) the transcripts of the mutated genes in a mixture containing up to 95% wild type mRNA, and (b) the newly acquired mutant genes in transgenic mice.

INTRODUCTION

In recent years recombinant DNA technology has dramatically improved our knowledge of eukaryotic gene structure and function, splicing (1,2), transcription initiation and its modulation (3-6), and transcription termination (7). Cell cultures and frog oocytes have become indispensable as host cells to test the engineered molecules, which can be transferred into the novel eukaryotic environment by a variety of techniques (8-11). In a number of laboratories, including our own, similar approaches have recently been extended to the transformation of entire organisms, in order to study gene regulation during development. The initial work in organism transformation was done using cloned genes derived from heterologous organisms partly to easily keep track of them in the host genome. Recombinant DNA molecules containing genes of SV40 (12), rabbit β -globin (13,14), human β -globin (15), human insulin (16) and herpes virus thymidine kinase (12,15,17,18) have been successfully introduced into living mice by microinjection into the zygote pronuclei and were found in some cases to be transmitted in a mendelian fashion to subsequent generations (14,16,18).

Analogous experiments carried out in our laboratory showed that rabbit globin-, SV40- and sea urchin histone genes can be transferred into *Xenopus laevis* fertilized eggs and maintained during development (19-21). Transformation with heterologous

genes as in the above mentioned experiments, has undoubtedly provided some general insights into the behavior of newly acquired DNA sequences in the host organisms. However, we believe that experiments of greater biological significance can be done by using homologous genes for transformation (see Discussion).

To avoid using heterologous genes, we have transformed mice with a modified mouse α -globin gene. This gene was mutated in vitro in its 3' untranslated coding region in order to follow the fate of both the gene and its transcripts in the transgenic organism. The globin protein is however expected to be unaltered and fully functional. In the present paper we show that the modified α -globin genes (a) are not significantly affected in their expression level, as assayed in mammalian cultured cell lines in a transient expression assay, (b) give rise to transcripts which can be readily distinguished from the wild-type gene transcripts by means of S1-nuclease mapping, and (c) can be identified in transgenic mice by virtue of their different restriction pattern.

MATERIALS AND METHODS

In vitro mutagenesis of the mouse α -globin gene

The phage containing the mouse α -globin gene: λ gtWESMal (22) was a gift from P. Leder (Bethesda, Md.). The 10.4 kb EcoRI insert of eukaryotic DNA was recloned into pBR327 (23) at the EcoRI site and this recombinant was named pawt. A 0.56 kb BamHI-XhoI fragment containing the 3' portion of the gene (see Fig.1a) was excised from pawt and subcloned into a smaller vector. This fragment was cut at the unique HaeIII site and

both halves were dephosphorylated with bacterial alkaline phosphatase. Religation was done in the presence of an equimolar amount of plasmid vector opened with Bam-HI and XhoI and a 10-fold molar excess of double stranded XbaI DNA linker (BRL). The majority of the recombinants obtained after transfection of the ligation products into HB101 contained the additional XbaI site at the expected position and were called pSUB α (X). An analogous procedure was used on the BamHI-XhoI fragment of pSUB α (X) to introduce a SacI linker into the AluI site two nucleotides downstream of the translation-termination codon, thus creating pSUB α (XS). The entire 10.4 kb mouse DNA segment was then reconstituted by substituting the native 0.56 kb BamHI-XhoI fragment with the "X" or "XS"-mutated homologs, giving rise to the recombinants called p α M(X) and P α M(XS), respectively. The DNA molecules used for transient expression assay consisted of the three versions of the α -globin gene (wild-type, "X" and "XS") recloned into the SV40-plasmid vector pMLE. pMLE is a pML-1 (24) derivative containing the SV40 enhancer-origin region (T. Gerster, unpublished results). These clones were called pE α (wt), pE α M(X) and pE α M(XS), respectively.

Plasmid DNA preparation and manipulation were performed according to standard cloning procedures (25-27).

Mice, eggs and micromanipulation

Mice of the outbred strain ICR/J (obtained from Tierspital Zürich) were kept on a 12 hrs-dark schedule (17.00-05.00). Females selected in proestrus were caged in groups of two to four with either fertile or vasectomized males to obtain egg donors and recipients, respectively. Females showing vaginal

plugs were separated the next morning and eggs were collected from 2pm to 4pm from donor females by puncturing the ampullae of extirpated oviducts. Removal of the cumulus cells was done at room temperature in presence of 0.2mg/ml of bovine testes Hyaluronidase (Sigma) dissolved into Hank's Balanced Salt solution (Gibco) supplemented with 4mg/ml Bovine Serum Albumin (Sigma) and 35mg/L of sodium pyruvate (Gibco).

Most of the further manipulations (including the transfer into foster mothers) were carried out as described by others (12-17) except for the microinjection which was performed at room temperature, in absence of a CO₂ atmosphere, and suspending the eggs in the salt-serum-albumin-pyruvate medium described above. Embryos were harvested at day 9-12 of gestation and stored at -20°C until used for DNA analysis. In one series the manipulated embryos were allowed to develop to term and DNA was extracted from the tail tip of newborn mice as described in (18).

Embryo DNA analysis, transient expression assay

3 to 5 µg of embryo DNA were digested with either SacI or XbaI, run on agarose gel, blotted to nitrocellulose (28) and hybridized to a nicktranslated pBR322 recombinant containing the 3 kb region of the α -gene indicated in Fig.1a (top). Control lanes contained either mouse DNA alone (from untransformed individuals) or supplemented with trace amounts (corresponding to 2 to 10 copies per haploid genome) of XS-modified α -globin gene sequences. Transient expression assays in tissue culture cells were carried out as described (29). Briefly, recombinant DNA containing either wild-type or mutated

globin-genes was transfected into human HeLa cells in culture using the calcium phosphate precipitation method (8). 36-48 h later, RNA extracted from the entire cell population was analyzed. Typically RNA extracted from about 1×10^6 transfected HeLa cells was hybridized to 1 ng of the endlabeled probe (3000 cpm). The 3' endlabeled X-DNA probe was obtained by filling up the protruding single stranded regions at the BstEII site located in the third exon (see Fig.1) in a DNA-polymerase (Klenow enzyme) reaction in presence of ^{32}P -dCTP (Amersham). The 0.4 kb BstEII-XhoI fragment was then purified by agarose gel electrophoresis. The 5' endlabeled 0.3 kb XbaI-BamHI fragment of XS-DNA was obtained upon kinase reaction at the newly introduced XbaI site (see Fig.1b). Control RNA (wild-type) was extracted from peripheral blood of healthy adult ICR/J mice. Other conditions for S1 nuclease analysis were as described in (30).

RESULTS

Transient expression of wild-type, X and XS-modified mouse α -globin genes

The procedures of in vitro mutagenesis of the 10.4 kilobases EcoRI mouse genomic fragment carrying the α -globin gene are illustrated in Fig.1 (for details see Materials and Methods). The XbaI and the SacI nucleotide octamer sequences were inserted 29, respectively 2 base pairs downstream of the translation termination codon. The modified genes containing one (XbaI, Fig.1a), respectively two (XbaI + SacI, Fig.1b) linker oligonucleotide sequences are referred to as X- and XS-

genes, respectively, in contrast to the unmodified wild-type or native gene. The corresponding transcription products will be referred to as X-, XS- and wild-type mRNA, respectively.

The expression level of the three versions of the gene (X, XS and wild-type) was tested in a transient expression assay in HeLa cells. In order to obtain detectable amounts of specific RNA it was necessary to reclone both altered and unaltered α -globin genes into a plasmid vector containing the SV40 origin of replication (31) and the SV40 transcriptional "enhancer" (29). S1 nuclease mapping indicated that the three versions produced similar amounts of α -globin specific transcripts when transfected under identical conditions. Fig.2 (lanes 5 and 9) is an example of RNA samples extracted from transfections with X-DNA or wild-type DNA, hybridized to a 3' endlabelled probe and treated with S1 nuclease. Bands of similar intensity are visible at the positions expected for the specifically protected fragments (216 nucleotides for X-RNA and 135 nucleotides for wild-type RNA, explained below). S1 nuclease mapping of the RNA 5' end (F. Weber, unpublished results) revealed that in every case the transcripts initiate at the same position as the blood cell RNA.

Furthermore, we tested whether the transcripts of the modified genes, X- and XS-RNA, could be identified in presence of excess blood cell mRNA. As mentioned above, the 3' end labelled X-DNA probe is useful for the identification of the transcripts of the X-type (see also Fig.1a). Such an X-RNA can hybridize without interruption to the transcribed strand of X-DNA from the labelled BstEII end throughout the newly introduced XbaI octanucleotide to the poly A tail addition site,

producing a 216 bp protected fragment as shown in lane 5 of Fig.2. The hybridization of the 3' endlabelled X-DNA probe with wild-type α -globin RNA molecule generates a small loop at the inserted Xba site which can be recognized by the S1-nuclease. In this case the protected labelled fragment is only 135 nucleotides long (BstEII to XbaI site) as shown in lane 9. Both the 216 and the 135 nucleotide long fragments are visible in lane 6 which represents a reaction where a 20 fold excess of blood-extracted (wild-type) mRNA was mixed with the X-RNA prior to hybridization. None of these specific bands are visible when no RNA (lane 7) or RNA extracted from mock-transfected HeLa cells (lane 8) are hybridized with the same 3' endlabelled probe.

The 5' endlabelled XS-DNA probe (see Fig.1b) is designed to recognize the transcripts from both the X- and the XS-genes. The former will protect a 34 nucleotide fragment from the labelled XbaI site to the SacI site which is absent in X-transcripts, and the latter will hybridize without interruption to the boundary of the third exon (172 nucleotides). Bands of the above mentioned size are visible in lane 3 and 2, where RNA obtained from cells transfected with X-, respectively XS-modified α -globin genes was hybridized to the 5' endlabelled XS-probe. Mixing in a 20-fold excess of wild-type (blood extracted) mRNA did not disturb the analysis, and as expected failed to produce analogous bands when hybridized separately with the same probe (lane 4). On the other hand, addition of a 100-fold excess of blood-extracted mRNA resulted in a reduction of the intensity of the 172 nucleotide long band, probably due to the near saturating hybridization

conditions (no longer in DNA excess). About 20 cpm of the BstEII endlabelled probe were mixed into the same reaction in order to visualize the presence of wild-type RNA and gave rise to the 135 nucleotide band visible in slot 1.

Taken together, the data obtained from transient expression indicate that the slightly altered α -globin genes can give rise to stable transcripts which are readily identified by S1 mapping in a mixture consisting of about 95% wild-type and 5% modified RNA. We are currently testing variations of the S1 nuclease assay in order to detect X- or XS-modified α -globin transcripts present at even lower concentrations.

Transplantation of the XS-modified α -globin genes into mouse embryos

As mentioned in the Introduction, the mutant α -globin genes (X and XS-variants) have a unique restriction pattern which can be exploited to discriminate them from wild-type genes. For all the injection experiments described in this article, we used the double-modified (XS) version cloned into a pBR327 vector. The best results were obtained using recombinant molecules carrying a single 10.4 kb insert.

In the latest series 4 out of 23 analysed individuals were found to contain the mutant α -globin gene. They were labelled in chronological order, T1, T2, T3 and T4 (T symbolizing "transformant"). Three of them (T1-T3) were analyzed before birth (day 9-12 of gestation). The fourth one, T4, belongs to a group of five which were allowed to develop to term. Southern analysis was performed mostly on SacI digested material, since

it was empirically found that it was easier to digest embryo DNA with this enzyme than with XbaI. In SacI digested DNA derived from untransformed mice (e.g. in lanes 2 & 5 of Fig.3) two hybridizing bands of 3 kb and 8 kb are visible. This indicates that the ICR/J strain used has a second non-allelic α -globin gene as reported for several other strains (32). Mice transformed with the XS-modified gene are expected to have two additional SacI DNA fragments hybridizing with the probe used: a unique one of 2.5 kb (see map on Fig.1, top) and one of 8 kb (including pBR sequences) which should co-migrate with the 8 kb fragment of the second endogenous α -globin gene. This was found as shown in Fig.3 (lanes 3,6,7 and 12).

After digestion with XbaI the endogenous genes remained at relatively high molecular weight positions (8 and 10 kb, lane 8 of Fig.3b). The newly acquired sequences were cleaved into a 5.5 kb fragment containing the plasmid, a 3.7 kb fragment containing the gene, and a 4.3 kb fragment containing the 3' flanking region. The 5.5 and the 3.7 kb fragment strongly hybridized to the probe used (see Materials and Methods) whereas the 4.3 kb fragment has only 270 nucleotides overlapping with the probe and gave a much weaker signal (see the reconstruction experiment in lane 9). The other fragments released by either SacI or XbaI digestion were not hybridizing to our probe (see Fig.1a, top).

The intensity of the ^{32}P signal of the 2.5 kb SacI and of the 3.7 XbaI bands can be directly compared to the intensity of the endogenous bands, thus allowing an estimate of the copy number of the newly acquired sequences. As shown in Fig.3, this copy number varies considerably from one transformant to

another. In the case of T3 and T2 there are one, and less than one copy per cell of the XS-gene, respectively, as judged from the SacI and XbaI specific bands appearing in lanes 6,7 and 10,11 of panel b. The two other individuals, T1 (panel a) and particularly T4 (panel c), contain more copies per cell of the injected sequences. As estimated from different exposures of the same autoradiograph (see lanes marked by *) T1 should contain more than 8-10 copies, and T4 more than 60 copies of the XS-modified α -globin DNA per cell. In none of the cases additional bands, revealing either rearrangements of the injected genes or junction fragments to host DNA, were visible. Although the injected DNA is most likely integrated into the host chromosomal DNA it is not surprising that no junction fragments have been detected: we did not use the entire 10.4 kb globin DNA segment as a radioactive probe because it also contains repetitive sequences found elsewhere in the mouse genome. Moreover, the many gene copies in transformants T1 and T4 may be organized as head-to-tail multimers with the junction fragments underrepresented with respect to the genes (see also 20). Further studies are under way to elucidate the structure of the persisting XS- α -globin genes and their possible expression in the host organism. For future experiments, emphasis will be on raising and breeding transformed mice. This will provide us with a virtually unlimited source of DNA and RNA for detailed analyses.

DISCUSSION

Transformation experiments with higher eukaryotes have already shown that it is possible to efficiently transfer many kinds of cloned DNA molecules into the genomes of living mice or frogs. So far, there has been no report indicating that the newly introduced genes are transcriptionally regulated in the same fashion as in the original host. This could be due to species incompatibility since in most of the cases the transforming DNA and the transgenic organism have been of a different species. The problem has been partly solved by the use of chimeric genes, where a heterologous tracer gene is fused to the putative regulatory region of a homologous gene (17,18, F. Costantini and E. Lacy, personal communication). Following this protocol, recombinant DNA molecules containing mouse regulatory sequences fused to a heterologous gene have been transferred into cultured cells (33) as well as living mice (17,18). In both cases the results are encouraging, indicating that the heterologous "tracer" can be put under control of homologous control sequences. Nevertheless, we believe that one should have maximal homology between the transforming gene and the host in order to eliminate any kind of species incompatibility. The ideal test system would be the transformation of a mouse carrying a genetic disease with the corresponding wild-type gene. Our approach of injecting a mutated mouse gene is close to the ideal situation. Insertion of DNA linkers has two advantages over other types of mutagenesis: (i) the linker changes the restriction pattern of

the mutated gene, and (ii) in the S1 nuclease assay wild-type mRNA gives a characteristic extra band which provides us with an internal positive control.

In our strategy of mutating the 3' untranslated region of the α -globin gene (Fig.1) we deliberately avoided any alteration which, to our knowledge, could impair the activity of the tested gene. Our results demonstrate that the introduced SacI and XbaI linker DNA does not interfere with the expression of this gene in a transient expression assay (Fig.2). It remains to be seen whether the modified genes function well in reticulocytes of a transformed organism.

Furthermore, we show that the use of appropriate ^{32}P DNA probes for S1 mapping allows the easy identification of the X and XS-transcripts in a mixture containing up to 95% wild-type α -globin mRNA sequences (Fig.2). As shown in Fig.3, we obtained four transformed mouse embryos after microinjection of the XS-modified α -globin gene into zygotes. As expected, the additional SacI and XbaI restriction sites were diagnostic for the presence of the newly acquired XS-modified globin genes. As far as DNA analysis is concerned, one might argue that the cotransferred plasmid DNA is also diagnostic for successful gene transfer. In an eukaryotic environment, however, plasmid sequences can disturb the activity of a linked gene (35,19,29). Therefore, we have begun to inject plasmid-free eukaryotic DNA sequences.

We do not know whether or not our modified α -globin genes were integrated into the α -globin locus of chromosome 11. Homologous integration seems unlikely in view of the fact that others have failed to detect integration of transforming genes

into their corresponding chromosomal locus, both in mammalian cultured cell lines and in transformed mice. In contrast, homologous integration is readily observed in transformation experiments with yeast, and it can even be exploited to replace ("transplace") a wild-type gene with its in vitro mutated counterpart (36). The small size of the yeast genome (about 100 times smaller than the mammalian genome) and the relatively large size of the DNA molecules used for the experiments mentioned above (about 3 kb) may be parameters which positively influence the frequency of homologous integration events. In order to obtain similar results when transforming mice, it may be necessary to deal with proportionately larger DNA molecules (hundreds of kb in length) if a similar size ratio between transforming DNA and target genome has to be maintained. As discussed by Scherer & Davis (36) the presence of middle repetitive sequences in the transforming DNA molecules could increase the frequency of undesired integration events. In transformations with large DNA fragments this problem of repetitive sequences seems unavoidable, due to their interspersed nature.

We have discussed elsewhere (20) that homologous integration may be difficult to obtain in a mitotic recombination system. During mitotic sister chromatid exchange or crossover, the transforming DNA may preferentially end up close to, or within heterochromatic regions of the host chromosomes, and the newly integrated gene may become subject to unpredictable position-variegation effects from the highly packaged heterochromatic flanking regions (see 20). In keeping with this, it has been found that the expression level of a

modified thymidine kinase gene varies enormously among siblings in 2nd and 3rd generation of transformed mice (18, A. Senear and R. Palmiter, personal communication). Furthermore, the relatively high frequency of unequal crossover in repetitive DNA (a common component of heterochromatic regions) could induce fluctuations in the copy number of the newly integrated gene and eventually its loss, as shown in the case of a leu2 gene integrated into the rRNA gene cluster in yeast(37). Unequal crossover could therefore induce head-to-tail formation of the integrated sequences. In fact, transforming DNA is often found in the form of head-to-tail tandem multimers within the host genome (14,17,18,19). It is tempting to speculate that these structures may preferentially arise when the integrated DNA is flanked by repetitive sequences.

Many aspects of organism transformation have yet to be elucidated. Some of the problems may be solved by achieving integration of the transforming DNA at the homologous position in the host chromosome, or by transforming vertebrates with extrachromosomal DNA entities, as it has been possible in yeast (38,39). Further studies are required to find out whether such steps are really necessary, or whether it is possible to obtain developmental regulation of a gene even without its integration into the homologous locus.

* * *

ACKNOWLEDGMENTS

We thank Dr. P.Leder (Bethesda, MD) for mouse α -globin DNA; Drs. R. Jaenisch (Hamburg), F.Costantini and E.Lacy (Oxford) for helpful advice in mouse embryo manipulation and for discussions; Drs. C.Weissmann and H.M. Eppenberger (Zürich). for giving us access to their micromanipulation facilities.

We are also grateful to Dr. M.L.Birnstiel, Dr. Strebel, J.Banerji, S.Oberholzer and L.Olson for valuable support, instructions in mouse breeding, critical reading, typing of the manuscript and for help in taking care of the mice, respectively.

A visit of S.R. to the laboratory of C.Graham (Oxford) was supported by a short-term EMBO fellowship. This work was supported by the Schweizerische Nationalfonds, Grant Nr. 3.687.0.80 and the Kanton Zürich.

- 1) Dierks, P., Wieringa, B., Marti, D., Reiser, J., van Ooyen, A., Meyer, F., Weber, H. and Weissmann, C. in: Developmental Biology Using Purified Genes, ICN-UCLA Symposia on Molecular & Cellular Biology, ed. D.D. Brown (New York: Academic Press) Vol. 23, 347-366 (1981)
- 2) Hamer, D.H. and Leder, P. Cell 18, 1299-1302 (1979)
- 3) Grosschedl, R. and Birnstiel, M.L. Proc. Natl. Acad. Sci. (USA) 77, 1432-1436 (1980)
- 4) Grosschedl, R. and Birnstiel, M.L. Proc. Natl. Acad. Sci. (USA) 79, 297-301. (1982)
- 5) Benoist, C. and Chambon, P. Nature 290, 304-310 (1981)
- 6) Mc Knight, S. and Kinsburg, R. Science 217, 316-324 (1982)
- 7) Pellicer, A., Wigler, M., Axel, R. and Silverstein, S. Cell 14, 133-141 (1978)
- 8) Diacumakos, E.G. in: Methods in Cell Biology, ed. D.M. Prescott (Academic Press) Vol. 7, pp 287-311 (1973)
- 9) Wong, T.K., Nicolau, C. and Hofschneider, P.H. Gene 10, 87-94 (1980)
- 10) Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. The EMBO Journal 1, 841-845 (1982)
- 12) Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, A. and Ruddle, F.H. Proc. Natl. Acad. Sci. (USA) 77, 7380-7384 (1980)

- 13) Wagner, T.E., Hoppe, P.C., Jollick, J.D., Scholl, D.R., Hodinka, R.L. and Gault, J.B. Proc. Natl. Acad. Sci. (USA) 78, 6376-6380 (1981)
- 14) Costantini, F. and Lacy, E. Nature 294, 92-94 (1981)
- 15) Wagner, E.F., Stewart, T.A. and Mintz, B. Proc. Natl. Acad. Sci. (USA) (1981)
- 16) Bürki, K. and Axel, U. The EMBO Journal 1, 127-131 (1982)
- 17) Brinster, R.L., Chen, H.Y., Trumbauer, M., Senear, A.W., Warren, R. and Palmiter, R.D. Cell 27, 223-231 (1981)
- 18) Palmiter, R.D., Chen, H.Y. and Brinster, R.L. Cell 29, 701-710 (1982)
- 19) Rusconi, S. and Schaffner, W. Proc. Natl. Acad. Sci. (USA) 78, 5051-5055 (1981)
- 20) Rusconi, S., and Schaffner, W. submitted to Nature
- 21) Bendig, M.M. Nature 292, 65-67 (1981)
- 22) Leder, A., Miller, H.I., Hamer, D.H., Seidman, J.G., Norman, B, Sullivan, M. and Leder, P. Proc. Natl. Acad. Sci. (USA) 75, 6187-6191 (1978)
- 23) Soberon, X., Covarrubias, L. and Bolivar, F. Gene 9, 287-305 (1980)
- 24) Lusky, M. and Botchan, M. Nature 293, 79-81 (1981)
- 25) Morrow, J.F., Cohen, S.N., Chang, A.C.Y., Boyer, H.W., Goodman, H.M. and Helling, R.B. Proc. Natl. Acad. Sci. (USA) 71, 3455-3459 (1974)

- 26) Telford, J.L., Kressmann, A., Koski, R.A., Grosschedl, R. Müller, F., Clarkson, S.G. and Birnstiel, M.L. Proc. Natl. Acad. Sci. (USA) 76, 2590-2594 (1979)
- 27) Birnboim, H.C. and Doly, J. Nucleic Acid Res. 7, 1513-1523 (1979)
- 28) Wahl, G.M., Stern, M. and Stark, G.R. Proc. Natl. Acad. Sci. (USA) 76, 3683-3687 (1979)
- 29) Banerji, J., Rusconi, S. and Schaffner, W. Cell 27, 299-308 (1981)
- 30) Weaver, R and Weissmann, C. Nucleic acid Res. 7, 1175-1193 (1979)
- 31) Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. Cell 27, 279-288 (1981)
- 32) Whitney, J.B. III, Martinell, J., Popp, R.A., Russel, L.B. and Anderson, W.F. Proc. Natl. Acad. Sci. (USA) 78, 7644-7647 (1981)
- 33) Chao, M.V., Mellon, P., Wold. B., Maniatis, T. and Axel, R. in: Proceedings of the Symp. on Hemoglobin Synthesis, University of Chicago, ed. E. Goldwasser (New York, Elsevier/North Hollynd) in Press, (1982)
- 34) Brinster, R.L., Chen, H.Y., Warren, R.L., Sarthy, A. and Palmiter, R. Nature 269, 39-42 (1982)
- 35) Probst, E., Kressmann, A. and Birnstiel, M.L. Jour. Mol. Biol. 135, 709-732 (1979)
- 36) Scherer, S. and Davis, R.W. Proc. Natl. Acad. Sci. (USA) 76, 4951-4955 (1979)
- 37) Szostak, J.W. and Wu, R. Nature 284, 426-430 (1980)

- 38) Beggs, J.D. Nature 275, 104-109 (1978)
- 39) Blanc, H., Gerbaud, C., Slonimski, P.P. and
Guerineau, M. Mol. Gen. Genet. 176, 335-342 (1979)

FIGURE 1 IN VITRO MUTAGENESIS OF THE MOUSE α -GLOBIN GENE.

Top, The *Xba*I and *Sac*I restriction maps of the 10.4 kb genomic DNA segment harboring the mouse α -globin gene (22) are shown.

Y^L indicate the positions at which the corresponding linkers were introduced. Other symbols: open bars, intervening sequences; arrow, direction of transcription; at the very top: MT , segment subcloned into pBR and used as a nicktranslated probe for the Southern hybridization shown in Fig. 3.

a) **wt DNA**, An expanded version of the 0.56 kb *Bam*HI-*Xho*I fragment containing the 3' portion of the gene. The *Hae*III restriction site used for in vitro mutagenesis is shown. Symbols: white bar second intervening sequence (IVS2); Black bars, translated regions; Thick line, 3' untranslated region; Thin line, flanking region downstream of the poly-A addition site. **X-DNA**, the same 0.56 kb fragment after the introduction of an *Xba*I linker octanucleotide (open thick line). The position of the *Bst*EII restriction site used for endlabeling is shown. The hybrid molecules formed with wild type mRNA and X-mRNA are also shown (wavy line, mRNA; straight line, DNA probe; * position of the ^{32}P -endlabeled). The numbers at the right (i.e. 135 and 216) indicate the size in nucleotides of the labeled S_1 -resistant fragments.

b) **XS-DNA**, The X-DNA fragment after introduction of the *Sac*I linker octanucleotide at the *Alu*I site located 2 base pairs downstream of the translation termination codon. The hybrid molecules formed with an *Xba*I 5' endlabeled XS-DNA probe and wild-type, X-, and XS-mRNA are shown. (Symbols as in (a)).

FIGURE 2 ANALYSIS OF THE RNA PRODUCED BY TRANSIENT EXPRESSION
OF THE THREE VERSIONS OF THE MOUSE α -GLOBIN GENE.

Top: a scheme indicating the RNA used on the hybridization reactions. The position of the circles indicates the type of RNA contained in each reaction (top, middle and lower corresponding to wild-type, X- and XS-RNA, respectively). The amount of RNA are also indicated: $\bigcirc$, RNA extracted from 10^6 HeLa cells transfected with the corresponding DNA recombinant; $\bullet$, addition of 20 and $\odot$, 100 fold excess of blood-extracted (wild-type) α -globin mRNA; $\star$, RNA extracted from 10^6 cells mock transfected with pBR322; lane M, molecular weight marker (HpaII endlabeled fragments from pBR322, (19)).

Polyacrylamide gel electrophoresis and autoradiography were carried out as described (19). Lanes 2-4 of the left panel show the S_1 -resistant fragments obtained using the 5'XbaI-endlabeled XS-DNA probe. (For lane 1, see text). The right panel shows the S_1 -resistant fragments obtained with the BstEII 3'-endlabeled X-DNA probe. The lines and the numbers in the center indicate the position and size (in nucleotides) of the S_1 -resistant fragments mentioned in the text. Migration of the DNA in slots 7,8 and 9 was progressively retarded (margin effect of the gel). The arrow in lane 9 indicates the position of the 135 nucleotide fragment.

FIGURE 3 TRANSFORMATION OF MICE WITH THE MUTANT XS- α -GLOBIN GENE

Symbols : R , slots showing the reconstruction experiments (see Materials and Methods); M ,molecular weight standard (λ phage DNA digested with EcoRI end HindIII, trace amounts of ^{32}P -nicktranslated phage DNA were added to the hybridization mix in panel (a)).; C , control mouse DNA (untransformed individual); T1 , T2 , T3 and T4 , DNA from transformed mice.

a), SacI-analysis of transformed individual T1. Lanes 3 and *, autoradiograph obtained from the same slot after an exposure time of 100 and 17 hours, respectively.

b), SacI- and XbaI-analysis of transformed individuals T2 and T3. Arrows and numbers indicate the position and the size in kb of the fragments mentioned in the text.

c), SacI-analysis of transformed individual T4. In analogy to (a): lanes 12 and * represent an exposure time of 120 and 1.5 hours, respectively.

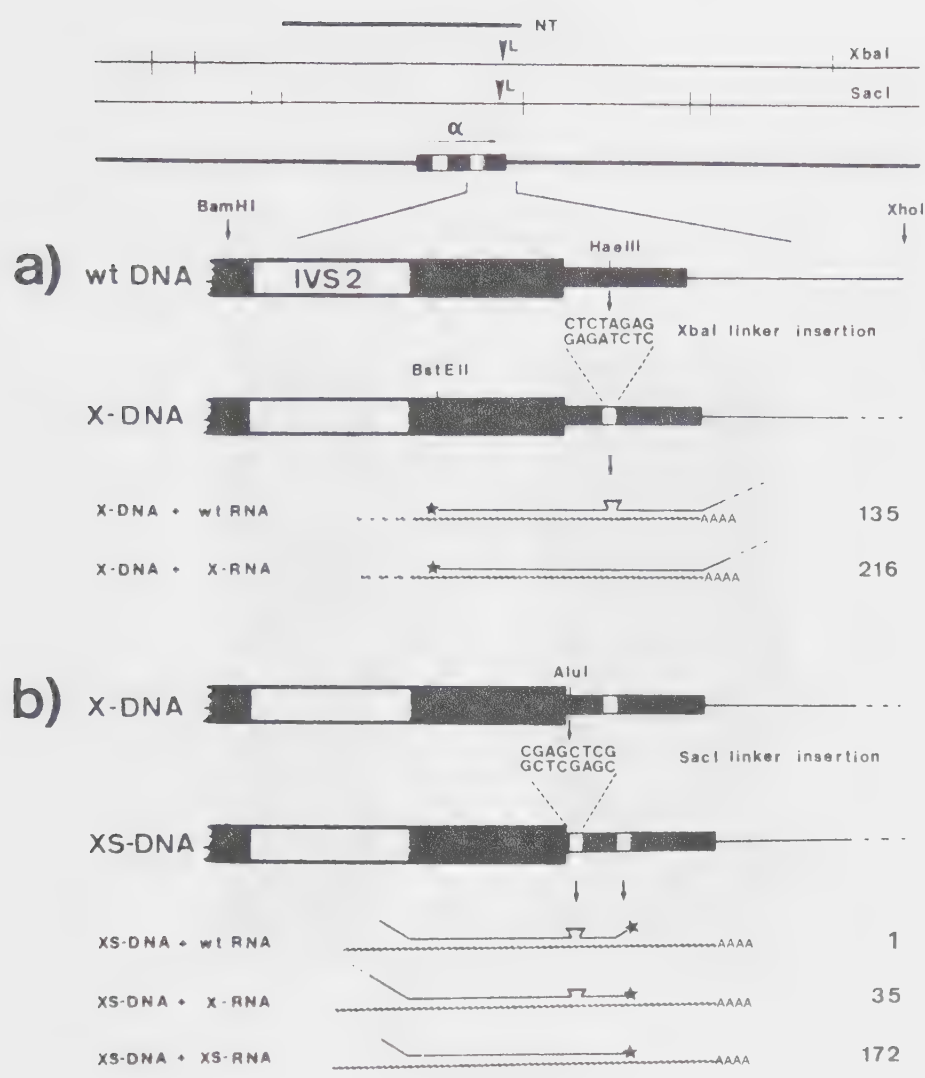


Figure 1

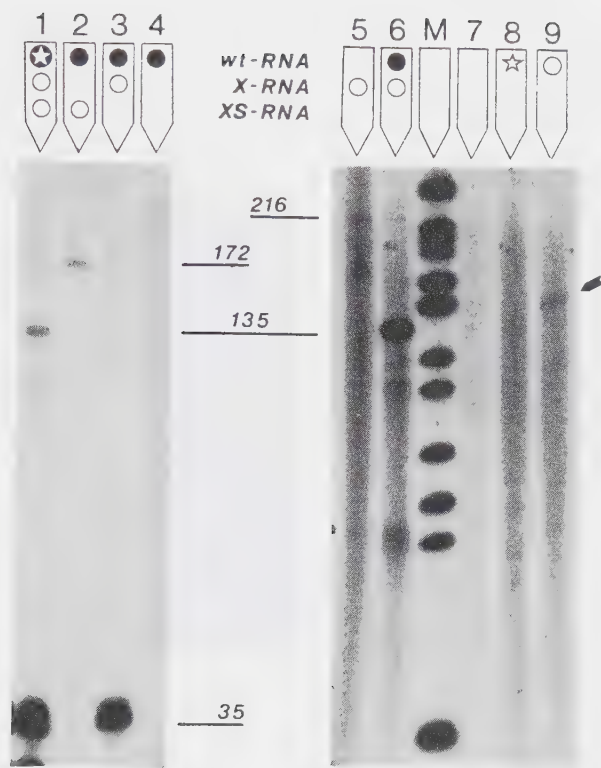


Figure 2

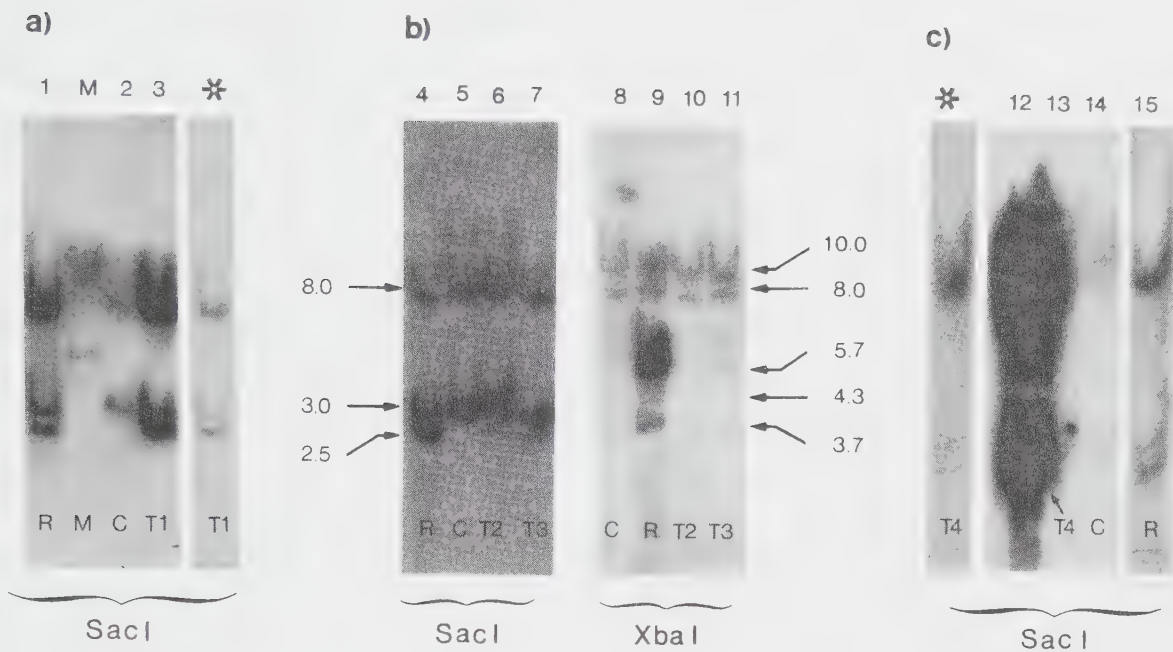


Figure 3

Zürich, den 3.8.82

C U R R I C U L U M V I T A E

von : Alessandro Rusconi
von : Locarno (CH, TI)
geboren : den 9. März 1952 in Muralto (TI)
Schulen : 1958-72 Schulen in Locarno (Lehrerseminar)
1972 Primarlehrerpatent (107/120)
1972-74 Lehrer in Locarno

Hochschule : 1974-76 Grundstudium im Hauptfach Biologie
mit Vorprüfungen (Universität ZH).
1976-77 Mittelbaustudium in der Fachrichtung
Molekularbiologie.

Verzeichnis der Dozenten ('74-'82):

E. Batschelet, R. Bachofen, J. Biegert,
M. Billeter, M.L. Birnstiel, E. Brun,
H. Burla, P.S. Chen, Ph. Christen, C.D.-
K. Cook, J. Deak, E. Dubler, A. Düben -
dorfer, P. Endress, Ch. Eugster, H. Fischer,
H.Hohl, R.Humbel, J.Kägi, E.Kuhn-Schny-
der, H.Kummer, P.Labhart, R.Nöthiger, H.
R. Oswald, D. Rast, H. Rieber, W. Schaffner,
M. Viscontini, G. Wagnière, H. Wan-
ner, H. Weber, R. Wehner, Ch. Weissmann,
V. Ziswiler .

1977-78 Vollpraktikum und Diplomarbeit in Moleku-
larbiologie. Thema : Isolierung und Cha-
rakterisierung von Histonvariantgenen des
Seeigels P.miliaris. Leitung: Prof. M.L.
Birnstiel und Dr. R. Portmann, Inst. für
Molekularbiologie II.

1979 Diplom in Molekularbiologie .

1979 (bis heute): Assistent am Institut für Mol.
biol.II und Ausführung der Dissertations-
Arbeit unter der Leitung von Prof.Schaffner.
Thema: Injektion klonierter Erb-
faktoren in befruchtete Wirbeltiereier.

